PhD Thesis

Behavioural and Physiological Stress Responses to Environmental and Human Factors in Different Small Mammal Species:

IMPLICATIONS FOR THEIR CONSERVATION

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UNIVERSIDAD AUTÓNOMA DE MADRID
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Memoria presentada por **Álvaro Navarro Castilla** para optar al grado de Doctor Europeo en Ciencias Biológicas por la Universidad Autónoma de Madrid

Bajo la dirección de la **Dra. Isabel Barja Núñez**, Profesora del Departamento de Biología de la Universidad Autónoma de Madrid

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A mi familia

A ti

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CONTENTS

ABSTRACT

Wild animal populations are continuously subjected to periodic disturbances by environmental and anthropogenic causes. Thereby, in this doctoral thesis, we carried out different investigations to study the behavioural and physiological stress responses of several small mammal species to different environmental and human factors. Behavioral responses to predation risk, interspecific competition and human disturbances were analyzed by live trapping. For analyzing the correspondent physiological stress responses, fresh faeces of individuals captured were collected during each experimental situation and the quantification of faecal corticosterone metabolites (FCM) as indicator of the adrenal activity was performed in the laboratory by an enzyme immunoassay. Results showed that wood mice (*Apodemus sylvaticus*) individuals responded to predation risk situations by recognizing and avoiding their main known predators in the study areas (they did not respond to the faecal odour of an unknown one) and also avoiding increased moonlight. Besides, foraging activity and food intake vary in relation to the risky situations but no physiological stress response was found due to the increase in predation risk. In relation to the interspecific competition with wild ungulates, Algerian mice (*Mus spretus*) abundances were higher inside areas where ungulates were not present being habitat selection influenced by greater vegetation cover. Increased FCM levels inside areas without ungulates were due to the higher mice abundance whereas in the grazed areas depended on the higher soil compaction caused by ungulates. Finally, we studied whether human disturbances through agricultural practices and motorways affected small mammal populations. We found a smaller number of common voles (*Microtus arvalis*) inhabiting inside crops, additionally, individuals captured inside crops showed higher FCM concentrations. In relation to the motorway, wood mice living closer to the motorway showed the highest FCM levels. Based on these results, we can conclude that small mammals generally respond by altering their behaviour when facing daily environmental or human disturbances. However, only interspecific competition with ungulates and human disturbance evoked physiological stress reactions whereas predation risk factors did not evoke such responses, suggesting that maybe these cues are not enough reliable to experience physiological stress responses since prey species daily face the risk of predation and therefore such continuous responses could be costly and pathological for individuals.

ABSTRACT

All the behavioural and physiological stress responses studied also depended on individual characteristics (e.g. sex, age and breeding condition) probably based on previous experience and the balance of costs-benefits in each particular situation at a given place and time. Since small mammals are good indicators of environmental impacts and they play an important role either as seed dispersers or as a major resource for many predators, hence, understanding how they cope with diverse difficulties could be crucial for developing effective management and conservation strategies in these and other free-ranging species.

Keywords: human disturbances, interspecific competition, physiological stress reactions, predation risk, small mammals

RESUMEN

Las poblaciones animales en estado natural se encuentran sometidas a perturbaciones periódicas debido a causas ambientales y humanas. Por tanto, en esta tesis doctoral, hemos llevado a cabo distintas investigaciones para estudiar las respuestas comportamentales y de estrés fisiológico en varias especies de micromamíferos a diferentes factores ambientales y humanos. Las respuestas comportamentales al riesgo de depredación, competencia interespecífica y perturbaciones humanas se analizaron mediante trampeos de vivo. Para analizar las respuestas de estrés fisiológico, se recolectaron heces frescas de los individuos capturados durante cada situación experimental y la cuantificación de metabolitos de corticosterona fecal (MCF) como indicador de la actividad adrenal se realizó en el laboratorio mediante enzimoinmunoensayos. Los resultados mostraron que el ratón de campo (*Apodemus sylvaticus*) respondía al riesgo de depredación reconociendo y evitando a sus principales depredadores conocidos en las áreas de estudio (no respondiendo al olor fecal de un depredador desconocido) y evitando también entrar en las trampas los días de mayor iluminación lunar. Además, la búsqueda e ingesta de alimento varió en relación a las distintas situaciones de riesgo pero no se encontraron respuestas de estrés fisiológico debido al aumento del riesgo de depredación. En relación a la competencia con ungulados salvajes, las abundancias de ratón moruno (*Mus spretus*) fueron mayores dentro de las áreas donde los ungulados no estaban presentes y la selección de hábitat estuvo relacionada con una mayor cobertura vegetal. El incremento en los niveles de MCF dentro de las áreas sin ungulados estuvo asociado a las mayores abundancias de ratón mientras que en las áreas con ungulados se relacionó con el incremento en la compactación del suelo causada por los ungulados. Finalmente, estudiamos si las perturbaciones humanas a través de las prácticas agrícolas y las autopistas afectaban a las poblaciones de micromamíferos. El número de capturas de topillo común (*Microtus arvalis*) dentro de los cultivos fue menor, además, aquellos individuos capturados en los cultivos mostraron mayores niveles de MCF. Respecto a las autopistas, los ratones de campo que vivían más cerca de la autopista mostraron concentraciones de MCF mayores. En base a estos resultados, podemos concluir que frente a las perturbaciones ambientales y humanas, los micromamíferos responden alterando su comportamiento.

RESUMEN

Sin embargo, sólo la competencia con ungulados silvestres y las perturbaciones humanas causaron respuestas de estrés fisiológico mientras que el riesgo de depredación no produjo tal respuesta, sugiriendo que quizás éstas señales no son suficientes para activar la respuesta de estrés fisiológico ya que las presas están diariamente sometidas a riesgo de depredación y por tanto estas respuestas serían muy costosas y patológicas para los individuos. Todas las respuestas comportamentales y de estrés fisiológico estudiadas también dependieron de las características individuales (p.e. sexo, edad y estado reproductor) probablemente basadas en la experiencia previa y el balance de los costes-beneficios en cada situación particular, en un lugar y momento determinados. Dado que los micromamíferos son buenos indicadores de los impactos ambientales y que tienen un papel importante tanto como dispersores de semillas así como por ser un recurso para muchos depredadores, comprender cómo pueden verse afectados ante diversas dificultades puede ser crucial para desarrollar planes de gestión y estrategias de conservación efectivas en éstas y otras especies.

Palabras clave: competencia interespecífica, micromamíferos, perturbaciones humanas, respuestas de estrés fisiológico, riesgo de depredación

INTRODUCTION

PREDATION RISK AND CHEMICAL COMMUNICATION

In nature, animals are exposed to a broad range of threats and dangers. Among these threatening factors, predation represents one of the most selective forces acting on the evolution of prey species (Yin et al. 2011). Predation is an important cause of death for many small mammals strongly influencing the ecology of prey species directly by killing them or indirectly by altering prey behaviour (sees Lima & Dill 1990).

According to Schmitz et al. (2013), predation involves at least five strategies: detection, identification, approach, subjugation, and consumption. Therefore, detecting a predator prior to a possible encounter is the first step of defense in the antipredatory strategy. Consequently, prey animals have developed different adaptations (e.g. morphological, behavioural, and physiological) that allow primary detection, recognition and avoidance to reduce the probability of encounters with their potential enemies (Lima 1998), as well as secondary defense mechanisms against predators (Luttbeg & Trussell 2013), for decreasing the risk of being predated.

Many carnivores use scents for marking their home range or territories by depositing urine, faeces or odorous glandular secretions (Brown & Macdonald 1985; Hutchings & White 2000; Barja 2009; Piñeiro et al. 2012). Although these scent markings possess an intraspecific means of interaction between conspecifics, they are also one of the main means of exchanging information at interspecific level (Gorman 1990). Thus, scent marking from carnivores may alert prey making possible to detect predator presence (Hughes et al. 2009). Hence, although visual and auditory cues are important, detection of mammalian predators is generally based on residual olfactory cues (Kats & Dill 1998). These chemical cues seem to be an accurate indicator providing useful information to the prey about the risk of predation, even in the absence of the predator (Mirza & Chivers 2003; Kusch et al. 2004).

Detection of these cues and predator recognition may depend on a number of factors. Prey species have been shown to detect and respond to a variety of cues associated with predators both in the field and in laboratory studies (Dickman & Doncaster 1984; Jedrzejewski et al. 1993; Apfelbach et al. 2005; Fendt 2006; Hegab et

al. 2014; Tortosa et al. 2015). The response of many prey species to predator odours seems to be a generalized avoidance behavior (Apfelbach et al. 2005) and it has been explained by the 'common constituents hypothesis' of Nolte et al. (1994). They suggested that odours of carnivores share common compounds that prey use as indicators of the amount of meat in a predator´s diet, allowing them to respond in a general way even to unknown potential predators. However, prey species might adjust this generalized response according to other components since some prey have shown predator-specific responses behaving differently when facing odours from different predators (Jedrzejewski et al. 1993). In addition, prior experience with predators seems to be a key factor. The 'prey naïveté hypothesis' of Cox & Lima (2006) predicted that some prey species may not respond to the odours of unknown predators if they have not been previously exposed to them due to the lack of a common historical evolution between both species (e.g. Dickman 1992; Mcevoy et al. 2008). Thus, the ability of prey to recognize and react to predator odours may depend on the life history, ecology and the evolutionary history of both predator and prey (Hayes et al. 2006b). However, predator olfactory recognition may need to be learned (Blumstein et al. 2002) and over time, prey species have demonstrated to be able to recognize and avoid new introduced predators (Russell & Banks 2007; Tortosa et al. 2015).

The increase in predation risk normally evokes different behavioural responses in prey species. Under risky situations, some small mammals usually alter activity patterns and space use (Lima & Dill 1990; Kats & Dill 1998; Jacob & Brown 2000). Thus, by reducing foraging activity (Jedrzejewski & Jedrzejewska 1990; Díaz et al. 2005), foraging more in covered microhabitats (Orrock et al. 2004; Fey et al. 2006) or forage more intensively close to refuges or escape routes (Brown & Morgan 1995; Thorson et al. 1998), prey reduce exposition to the risk of an encounter with a predator. On the other hand, environmental conditions such as cloud cover, rainfall or lack of moonlight lead to an increase in foraging activity since these conditions make prey safer and less detectable by predators (Kotler et al. 2010; Wróbel & Bogdziewicz 2015). Indeed, in some circumstances, small mammals seem to alter their foraging activity in response to indirect cues (e.g. dangerous microhabitats or weather conditions) rather than to direct ones (e.g. odours or sounds) produced by the predators (Orrock et al. 2004)(Blumstein et al. 2002; Orrock et al. 2004). Thus, indirect cues probably provide

truly accurate information about the risk from multiple predators (Blumstein et al. 2002).

Although antipredatory strategies are crucial for prey survival, they involve important associated costs. Increasing antipredatory behaviours leads to a decrease or stop of other non-defensive behaviours like feeding or breeding (Koivisto & Pusenius 2003; Brown & Kotler 2004; Preisser et al. 2005) even affecting individual fitness (Lima & Dill 1990). Thus, according to the risk allocation model proposed by Lima & Bednekoff (1999), animals should increase their foraging effort under low predation risk circumstances whereas they should decrease it during high risk situations. However, if animals are exposed to high risk during long periods, they will need to resume foraging under these situations to meet their energy requirements. Therefore, preys should balance the time dedicated to antipredator responses and other non-defensive daily activities in relation to temporal variation of the risk of predation perceived (Lima & Bednekoff 1999). In conclusion, since behavioral anti-predator responses involve tradeoffs and costs, they seem to be limited to situations which they are truly effective (May et al. 2012). Hence, behavioral antipredator responses are expected to be used only when an individual has a precise evaluation of the existing predation risk, and the benefits of such response must outweigh the associated costs (Dielenberg & McGregor 2001; Kavaliers & Choleris 2001).

INTERSPECIFIC COMPETITION

Milne (1961) defined competition among animals as "*the endeavour of two (or more) animals to gain the same particular thing, or to gain the measure each wants from the supply of a thing when that supply is not sufficient for both (or all*)". Since all animals require resources to grow, reproduce and survive, competition is commonly used to refer to a negative relationship or interaction occurring between individuals, of the same or different species, whenever two or more individuals require the same limited resource (e.g. food, water, refuge, mate or territory). Therefore, competition is harmful for both or all parties and individuals are adversely affected by those having the same requirements.

There are two different types of competition, interference and exploitative competition. If competing individuals interact directly by physical interactions (e.g. fighting) for limited resources, this is defined as interference competition, whereas

exploitative competition occurs when individuals interact indirectly for example consuming the same scarce resources so they become no longer available for others. In addition, when competition happens among individuals of the same species it is called intraspecific competition (Hansen et al. 1999; Koivisto et al. 2007)(Hansen et al. 1999; Koivisto et al. 2007), while if members of different species compete for the same limiting resources it is known as interspecific competition (Connell 1983; Schoener 1983; Koivisto et al. 2008).

Interspecific competition is an important biological factor with a huge effect on animal communities. Thus, interspecific interactions between small mammal species have been widely studied showing negative effects on population growth, survival or reproductive success for the species involved (Eccard & Ylönen 2002; Glazier & Eckert 2002; Eccard & Ylönen 2003; Koivisto et al. 2007). However, these competitive interactions between small mammal species can be altered by other external factors such as predation, which can reduce direct competition between small mammal species or even benefit to one species (Gurevitch et al. 2000; Chase et al. 2002; Koivisto et al. 2008). Likewise, ungulates have been shown to influence small mammal interactions (Steen et al. 2005) being also potential competitors for them. Although interference interactions between small mammals and ungulates are unlikely, however, exploitation competition occurs due to diet and habitat overlap and because of the limiting shared resources (Tokeshi 1999). Thus, small mammals are affected by grazing which reduces vegetation cover increasing predation exposure (Schmidt & Olsen 2003; Orrock et al. 2004), physical disturbance due to trampling of soil, browsing and rooting (Hayward et al. 1997; Focardi et al. 2000; Torre et al. 2007) and by competing with ungulates for the same food resources (Keesing 1998; Schmidt et al. 2005). Nevertheless, although small mammals can be affected by ungulates the effect of the interactions seems to depend on ungulates densities (Steen et al. 2005). In addition, small mammal species have been shown to respond differently (i.e. showing increases or decreases in population size) to ungulates (Keesing 1998; Jones & Longland 1999; Giuliano & Homyack 2004; Steen et al. 2005). Hence, interspecific competition with ungulates potentially alters the balance of competition between small mammal species influencing community dynamics.

HUMAN DISTURBANCES

Practically all ecosystems are subjected to periodic natural disturbances but also to anthropogenic perturbations. Human disturbances lead to different alterations in the composition, structure and functioning of natural ecosystems (Rapport & Whitford 1999). The effects of these human caused disturbances have dramatic consequences in ecosystems on both local and global scales (Acevedo-Whitehouse & Duffus 2009), resulting in potentially negative effects on wildlife. Human intervention has greatly modified and decreased large natural areas worldwide being an important cause of biodiversity loss (Benton et al. 2003; Alkemade et al. 2009). Consequently, the extent and adverse consequences of human disturbances on wild animal populations are continuous issues in conservation biology.

Anthropogenic disturbances have resulted in increasingly severe perturbations evoking different responses upon animal populations. Animals mainly respond by avoiding humans since they perceive them as potential predators (Frid & Dill 2002), thus, human disturbance usually reduces access to resources because animals tend to avoid those areas affected by human activities or presence (Trombulak & Frissell 2000). In addition, other detrimental effects like reducing reproductive success and survival have been also reported due to human disturbances (Jacob & Halle 2001; Kerley et al. 2002). Species with large spatial requirements (e.g. carnivores) seem to be more vulnerable to human disturbances since they have to deal daily with human infrastructure and activities within their home ranges (Kerley et al. 2002; Martin et al. 2010), however, small mammals can be strongly affected by human disturbances (Sauvajot et al. 1998; Wu & Fu 2008). Thereby, even a simple outdoor recreational activity such as watching wildlife, which has increased rapidly worldwide (Giannecchini 1993), has been reported to cause negative effects on different wild animal populations including small mammal species (Boyle & Samson 1985; Stephenson 1993; Lott & McCoy 1995). Hence, if small mammals can be affected by the increased pressure from the ecotourism industry, there is little doubt that they could be also affected by other human made interventions which cause habitat loss and fragmentation, representing a major impact on natural ecosystems leading to worse consequences in biodiversity (Fahrig 2003).

In this regard, agricultural uses would be a good example since they represent the major land use in Europe and it is expected to continue growing due to food demand (Tilman et al. 2002; Green et al. 2005). To meet this demand, the strategy has been, and remains, increasing production through the transformation of natural environments into agricultural landscapes by irrigation, mechanization and application of fertilizers and pesticides. However, this conversion involves drastic changes in farming practices and in the farmland habitat and landscape, since it reduces vegetation boundaries, changes the types and sizes of crops and increases the use of fertilizers and biocides (Herrero & Snyder 1997). Different studies have reported agriculture affecting the composition and abundance of species (Robinson & Sutherland 2002; Benton et al. 2003; Brotons et al. 2004), therefore, it has been considered as a major cause of biodiversity loss and global change. Indeed, irrigation has been considered one of the greatest threats to the conservation of several species (Brotons et al. 2004). Small mammals are commonly found inhabiting in agricultural landscapes (Freemark 1995; Singleton et al. 1999) and they have been reported to be seriously affected by most farming practices (Freemark 1995; Jacob & Hempel 2003; Jacob & Hempel 2003). Agricultural practices are human-caused perturbations involving the modification of the original natural environments into degraded habitats which are continuously exposed to different alterations like tilling, harvesting or crop rotation (Ammann 2004). These periodic changes in habitat conditions affect the availability of resources (e.g. food, cover, refuges and nesting sites) for the small mammals. Main effects of farming practices have been demonstrated on population dynamics where several studies showed how after farming practices (e.g. ploughing, harvest, or mowing) population numbers decreased for different small mammals species (Lemen & Clausen 1984; Jacob & Halle 2001; Jacob & Hempel 2003). In addition, farming practices can caused also indirect effects by removing food and cover which exposes small mammals to an increase in predation risk (Sheffield et al. 2001; Orrock et al. 2004). Consequently, they have been reported to have an effect on the spatial behaviour for some small mammal species (Tew & Macdonald 1993; Jacob & Hempel 2003). Hence, keeping ecological compensation areas (e.g. natural field margins and herbaceous strips) in intensively farmed landscapes is of high importance for small mammals and overall to maintain biodiversity in agricultural landscapes (Aschwanden et al. 2007).

Another well-known and widespread human impact is the modification and fragmentation of natural habitats throughout the construction and maintenance of roads and motorways (Spellerberg 2002; Forman et al. 2003). This extensive network of linear infrastructures has been recognized as a major factor of biodiversity loss (Alkemade et al. 2009). Roads and motorways have different effects on wildlife (Trombulak & Frissell 2000). Direct effects are caused by the mortality from road construction and from collision with vehicles (Lodé 2000; Clevenger et al. 2003). These transport infrastructures lead to a higher use of natural areas by humans what can be translated into higher human disturbance and increased hunting and poaching activities (Trombulak & Frissell 2000; Kerley et al. 2002). Furthermore, they cause detrimental effects due to the alteration of the natural environment by traffic light, pollution and noise (van der Ree et al. 2011). Animals can be also indirectly affected due to habitat loss and fragmentation since these alterations represent a barrier effect (Klein 1971) clearly reducing the connectivity of the landscape (Saunders et al. 2002; Coffin 2007). This reduction inhibits crossing road movements leading to smaller isolated populations (Keller & Waller 2002). Therefore, both road kill and road avoidance may fragment and reduce wild animal populations until become locally extinct under some circumstances (Trombulak & Frissell 2000). Most of these studies have been done on medium and large mammals (Jones 2000; Lodé 2000; Kerley et al. 2002; Grilo et al. 2009) while fewer studies on the effects of roads have carried out on small mammal species since they are thought to be less affected because they have more possibilities to cross them through different mitigation measures (Dodd et al. 2004). Nevertheless, several studies have demonstrated that small mammals are also affected by these linear infrastructures since they act as physical barriers completely or partially inhibiting road crossings (Gerlach & Musolf 2000; Clark et al. 2001; Goosem 2001; Rico et al. 2007). Overall, the presence of roads is highly correlated with changes in species composition and population sizes (Trombulak & Frissell 2000). However, not all species are equally affected by roads, in fact, some small mammal species showed either positive effects or no effects (Fahrig & Rytwinski 2009). In addition, crossing inhibition has been shown to depend on road features (e.g. width) and species-specific behavioural responses (Goosem 2001; Rico et al. 2007).

Predicting the consequences of human disturbances and alterations in natural ecosystems is an important ecological issue since both natural and human disturbances rarely occur in isolation, and they may also interact influencing community structure and dynamics in ways not predictable (Sih et al. 2004). In natural ecosystems, small mammals are good indicators of environmental impacts (Steele et al. 1984) and they play an important role either as seed dispersers or as a major resource for many predators. Hence, the effects of human disturbances on the structure and dynamics of their populations may have detrimental cascading effects on other species. Therefore, since the impact of human activities and the negative consequent environmental changes may strongly affect the persistence of wild animal populations, understanding the effects of human disturbance on wildlife populations is critical for an effective management and their conservation.

PHYSIOLOGICAL STRESS RESPONSES

Studies on the response of wildlife to different environmental or human factors generally have focused on demographic or behavioural changes (see above). However, the reaction and even the quantity and quality of the effect can be strongly species-specific, even appearing individuals showing confused or no behavioural effects (Gill et al. 2001). In this regard, under certain situations animals can display a physiological stress response that is not translated into a behavioural change (Eilam et al. 1999).

Physiological stress can be defined as an adaptive response of an organism to a demand made by its environment (Möstl & Palme 2002). The event or force that evokes this response is called stressor, which can be physical (e.g. aggression, heat or cold, injury, environmental alterations) or psychological (e.g. fear, anger, anxiety or frustration). In response to a stressor, an animal mounts a stress response which includes a series of physiological and behavioural changes which serve to neutralize the effects of the stressor and to reestablish homeostasis (Möstl & Palme 2002). Among the physiological stress responses, when an animal is subjected to a stressor the endocrine stress response enhances the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. The HPA axis stimulates the hypothalamus to secrete corticotropine-releasing factor (CRF) inducing the anterior pituitary to release the adrenocorticotropic hormone (ACTH), which signals the adrenal cortex to increase the secretion of steroid hormones such as glucocorticoids (GCs; generally cortisol or corticosterone depending

on the species) to help the animals to overcome a stressful situation (Sapolsky et al. 2000; Melmed & Kleinberg 2003; Stewart 2003). Later, when the stressor decreases or ceases, a negative feedback response inhibits the secretion of GCs (Sapolsky et al. 2000). Generally, short-term GCs secretion last only a few hours and promotes successful adaptive responses to the stressful stimulus (Wingfield & Romero 2001), whereas chronic stress occurs when individuals experienced either multiple, frequent exposure to stressors, and/or long-term continuous exposure to stressors which generates elevated and prolonged high GCs levels exceeding the individual level of beneficial adaptation. Thus, increased GCs levels have been reported to potentially cause a wide range of pathological consequences including inhibition of growth, tissue atrophy, reproduction failure and immune function suppression (Munck et al. 1984; Sapolsky 1992; Sapolsky et al. 2000; Sapolsky 2002; Stewart 2003; Romero 2004). Many of these detrimental consequences have the capacity to affect the long-term persistence of natural populations since they affect resistance to diseases increasing animal´s vulnerability also compromising their reproduction and survival (Lochmiller & Deerenberg 2000; Berga 2008), which may affect fitness of individuals (Möstl & Palme 2002; Bonier et al. 2009).

GCs levels are used as hormonal indicators of physiological stress (Wingfield & Romero 2001; Möstl & Palme 2002; Barja et al. 2007) and they can be quantified in plasma, urine, saliva and faeces. GC levels assessment in blood plasma samples has been one of the accepted indexes of quantitative measurements of physiological stress conditions (Broom & Johnson 1993). However, this technique requires trapping, handling, sedation and/or transport and puncture (Graham & Brown 1996; Brown & Wildt 1997; Dehnhard et al. 2001; von der Ohe et al. 2004; Young et al. 2004), being and invasive method which may cause a possible induction of stress responses associated with sampling procedures (Cook et al. 2000; Place & Kenagy 2000; Vachon & Moreau 2001). Alternatively, faecal glucocorticoids (FGs) quantification provides a non-invasive measurement of stress avoiding the above mentioned logistical difficulties. This method has been widely used proving to be a valuable tool for evaluating stress physiology responses and endocrine status in a variety of species (Monfort et al. 1997; Palme et al. 1998; Goymann 2005; Touma & Palme 2005; Siswanto et al. 2008; Soto-Gamboa et al. 2009; Tarjuelo et al. 2015). Besides, contrary to serum sampling,

faecal samples for analyzing FGs are relatively easy to collect without disturbing animals (Monfort et al. 1998; Barja et al. 2012). This method has been applied for investigate adrenocortical activity during exposure to stressful stimuli in an increasing number of mammalian species (e.g. (Millspaugh et al. 2001; von der Ohe et al. 2004; Barja et al. 2008) including small mammals (Harper & Austad 2000; Hayssen et al. 2002; Good et al. 2003).

In mammals, a large body of experimental evidence has shown environmental and human factors causing physiological stress responses in wild fauna. For example, predation risk has been reported to evoke physiological stress responses in some prey species (Boonstra et al. 1998; Eilam et al. 1999; Monclús et al. 2009). Population densities and group size have been also correlated with increased physiological stress responses in different species (Rogovin et al. 2003; Pereira et al. 2006; Dantzer et al. 2013). Furthermore, physiological stress responses have been documented to vary in relation to diurnal (Ferreira Raminelli et al. 2001; Touma et al. 2003) and seasonal changes (Harper & Austad 2001; Girard-Buttoz et al. 2009; Carnegie et al. 2011). Likewise, different studies have demonstrated physiological stress responses to human disturbances on wildlife. Thus, continuous development of outdoor recreational activities (e.g. tourism, winter sports, logging and hunting) evoked pronounced physiological stress responses in a number of mammalian species (Bateson & Bradshaw 1997; Creel et al. 2002; Pereira et al. 2006; Barja et al. 2007; Burke et al. 2008; Piñeiro et al. 2012; Rimbach et al. 2013; Zwijacz-Kozica et al. 2013).

Responding to a stressor requires an animal to expend energy, which alters metabolic pathways for the production of ATP to generate extra energy to deal with the stressful situation (Creel 2001). Therefore, the impact of an aversive stressor is determined by the ability of an individual to cope with such situation, and the magnitude of the physiological stress response is subjected to individual variation, and can even vary over time within an individual. GCs levels can be also influenced by diverse factors (e.g. sex differences, breeding condition and social ranking) and physiological stress responses in free-ranging animals have been reported to vary by the life-history stage of individuals (Touma et al. 2003; Millspaugh & Washburn 2004; Keay et al. 2006; Mooring et al. 2006; Nováková et al. 2008; Carnegie et al. 2011; Ebensperger et al. 2011; Goymann 2012). Therefore, these individual variations must be

taken into account for establishing proper protocols for the study of physiological stress responses in wild animals exposed to environmental and human perturbations.

Stressors may be inevitable components in natural habitats, identifying environmental and human factors associated with increased GCs levels in wild fauna could help to foresee other changes with a slower response in time (e.g. apparent declines of populations or behavioural changes). Since physiological stress responses have a strong influence on health and survival, affecting also animal fitness (Möstl & Palme 2002), non-invasive GCs measurements are an important means to monitor wild animal populations providing accurate data concerning animal welfare, enabling to optimize management strategies and increasing the likelihood of conservation success.

STUDY SPECIES - SMMAL MAMMALS

Small mammals do not exist as a zoological group. This term has not taxonomic value but is generally used in research publications to refer to small sized mammal species. There is no a particular definition for the small mammal, for example Barnett & Dutton (1995) considered any non-flying mammal weighing less than 1 kg, while other authors have settled on including any mammal species weighing about 5 kg or less (Stoddart 1979; Degen 1997; Merritt 2010). In terms of number of species and individuals, small mammals dominate the Class Mammalia mainly including individuals from Lipotyphla (covers all the families that were considered to make up the traditional mammalian order Insectivora), Chyroptera, Rodentia, Lagomorpha and Carnivora orders, depending on authors (Stoddart 1979; Barnett & Dutton 1995; Pearch 2011).

Small mammals are unique in how they cope with a variety of environments. They are found in practically all habitats contributing to the biodiversity, both to the diversity of species and life forms present and to the functional diversity of the ecosystem. Small mammals are generally predated by avian and mammalian predators (Halle 1988; Halle 1993; Goldyn et al. 2003; Rosellini et al. 2008). In addition, small mammals consume invertebrates, plants, seeds and fruits (Hansson 1985; Fedriani 2005; Khammes & Aulagnier 2007), being also important disseminators of seeds (Pulido & Díaz 2005; Muñoz et al. 2009). Therefore, due to their important role in the ecosystems, small mammals can be good ecological indicators and they are considered as model

organisms for research studies. Thus, in this thesis we have carried out different experiments studying the following small mammal species:

Wood mouse (*Apodemus sylvaticus* Linnaeus, 1758)

Also known as the long-tailed field mouse, this commensal rodent belongs to the family *Muridae*, subfamily *Murinae* (Wilson & Reeder 2005). It occupies a large range throughout Europe (except in Finland and the northern parts of Scandinavia, the Baltic and Russia) and North Africa (Montgomery 1999; Wilson & Reeder 2005). It is a very adaptable species occurring from sea level to 3,300 m and inhabiting a wide variety of semi-natural, suburban and urban habitats mainly exploiting woodland, sand dunes, grassland, gardens and farmland (Zhang & Usher 1991; Gorman et al. 1993; Montgomery 1999).

In the Iberian Peninsula, the wood mouse is the one of the most abundant and widely distributed small mammal species (Alcántara & Tellería 1991; Torre et al. 2002). It shows golden-brown colour and relatively larger ears, eyes and tail, not exceeding 40 g (Blanco 1998). They are mainly active during the dark, usually picking up and distributing visually conspicuous objects (e.g. leaves and twigs) which they then use as landmarks during foraging and exploration (Stopka & Macdonald 2003). The wood mouse has a breeding season from February to October, in which multiple mating occur between males and females. The gestation period last 25–26 days, the offspring become independent after about three weeks and become sexually active after two months. The annual cycle of wood mice includes summer decline and autumn collapse of population density (Fons & Saint Girons 1993) and population density may fluctuate between years, but there are no regular cycles (Montgomery 1999). Sometimes this species can cause occasional damage (Pelz 1989), but it is not generally considered to be a pest species (Montgomery 1999).

Algerian mouse (*Mus spretus* Lataste, 1883)

The Algerian mouse belongs to the family *Muridae*, subfamily *Murinae* (Wilson & Reeder 2005) and it is a non-commensal rodent commonly named western Mediterranean mouse. This species is endemic to the Mediterranean region distributed in south-west Europe (from Portugal, across all but the northern fringe of the Iberian Peninsula up to southern France) and in North Africa occupying the Maghreb area from

Morocco up to Algeria, Tunisia and to northern Lybia (Panteleyev 1998; Macholan 1999).

The Algerian mouse has brownish fur over most of the body with distinct white or buff underparts. It presents small eyes and ears, a short tail and a body weight of less than 20 g (Palomo et al. 2009). Its vertical range is from sea level to 1,800 m, preferring a variety of open habitats such as orchards, arable lands, grasslands or dry scrublands (Orsini et al. 1982; Blanco 1998; Palomo et al. 2009). However, it is absent from dense forests and it is considered a fully wild species avoiding human settlements (Macholan 1999), but it may occasionally found in abandoned buildings (Palomo et al. 2009). The Algerian mouse is primarily nocturnal. It is an opportunistic omnivore, primarily feeding on grass seeds, fruit, and insects (Orsini et al. 1982; Khidas et al. 2002). Its diet reflects the availability of resources more than its preferences for certain food items. Algerian mice breed for nine months of the year being sexually inactive from November to January (Vargas et al. 1991). Gestation lasts 19-20 days and the young reach full adult size at eight to nine weeks, being already sexually mature (see Palomo et al. 2009). The population structure varies throughout the year, according to reproductive cycle, but it is not considered a pest species.

Common vole (*Microtus arvalis* Pallas, 1778)

The common vole belongs to the family *Cricetidae*, subfamily *Arvicolinae* (Wilson & Reeder 2005) and it is the most abundant small mammal species in Europe (Mitchell-Jones et al. 1999). This rodent shows an extending range from Northern Spain, across continental Europe up to the Middle East and central Russia (Haynes et al. 2003; Shenbrot & Krasnov 2005). The species occurs from sea level to about 2,600 existing in diverse habitats. It is found in primary habitats such as grasslands meadows, flower strips, field margins and set-aside and fallow lands, as well as in secondary habitats like agricultural areas mainly including grain cereals, sugar beet and potatoes crops (Mitchell-Jones et al. 1999; Le Louarn & Quéré 2003).

An adult common vole weighs about 25-30 g, reaching more than 50 g in males and 40 in females under optimal conditions (Wilson & Reeder 2005). They live in shallow burrows of 30-50 cm deep (Brügger et al. 2010) which tunnels help to soil aeration and fertilization also providing shelter for many other species (Martin 2003). Common voles can be active during day and night (Daan & Slopsema 1978) and their

diet is based on a large number of plant species, seeds and roots (Rinke 1990). They breed mainly from April to November (Jacob 2000) and the highest population densities are usually achieved typically in autumn. After a tree week gestation period, common voles can begin reproducing at an early age of 14 days (Tkadlec & Zejda 1995). Litter sizes can reach more than ten pups, with an average of 4.5 litters produced per breeding season (Boyce & Boyce 1988; Boyce & Boyce III 1988). Due to its high reproduction rates and its plant-based diet, the common vole is considered a pest species since outbreaks occur about every 2-5 years (Cornulier et al. 2013) reaching densities of over 2,000 individuals in peak years (Bryja et al. 2005) and causing significant economic losses due to crop damage (Olea et al. 2009; Jacob & Tkadlec 2010).

RESEARCH AIMS

The main purpose of this thesis was to study those environmental and human factors evoking behavioural responses and potentially affecting the physiological status of wild populations. To do so, we examined the effects of predation risk and interspecific competition as environmental problems and the effects of agricultural practices and motorways as human disturbances, on behavioural and physiological stress responses of different small mammal species (wood mouse *Apodemus sylvaticus*, Algerian mouse *Mus spretus* and common vole *Microtus arvalis*) as model organisms.

In particular, we studied chemical recognition of different mammalian predators by wood mice and the effects of predation risk factors on this prey species (Chapter I). We predicted that wood mice would better recognize and avoid their main natural predators in the study area, the red fox *Vulpes vulpes* and the common genet *Genetta genetta*, but not an unknown one, the European pine marten *Martes martes* (Paper I). Based in these previous findings, we tested the behavioral and physiological stress responses of wood mice by experimentally exposing them to: i) the faecal odour of red fox and common genet and ii) increased moonlight illumination (Paper II and Paper III). Since predator cues inform of a possible imminent attack and increased illumination make preys more vulnerable to predators, we predicted wood mice avoiding these risky situations. In addition, under predation risk wood mice would reduce foraging and feeding behaviour, and they are also expected to show a noticeable physiological stress response. If so, predation risk could be directly influencing population densities and wood mice behaviour, but also potentially affect their physiological status with the correspondent negative consequences for them.

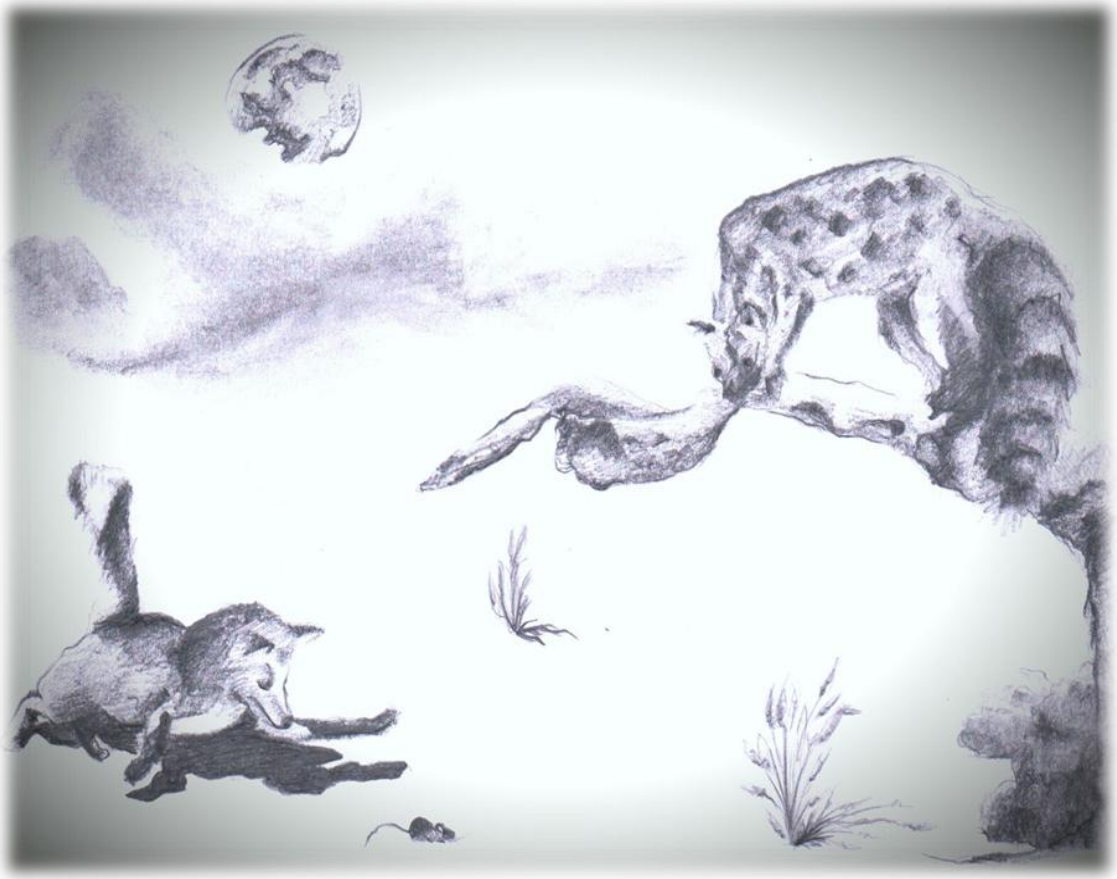
In Chaper II, we tested whether interspecific competition with wild ungulates would result in behavioral and physiological stress responses in the Algerian mouse by studying these responses in different grazed and no grazed (exclosures) areas (Paper IV). Taking into account that in the study area, and specially during some times of the year, Algerian mice compete with ungulates for food resources, and additionally, grazing and browsing by ungulates also reduce cover and refuges for Algerian mice. Therefore, we predicted that Algerian mice would respond by avoiding grazed areas and we expected increased physiological stress responses in those individuals subjected to ungulate pressure in the grazed areas.

Finally, in Chapter III, we aim to evaluate the effects of different human disturbances on small mammal populations. We studied the behavioral and physiological response of common voles to degraded habitats due to the agricultural practices (Paper V). We predicted that common vole abundances would be higher outside crops (in the natural field margins) and those individuals inhabiting inside the agricultural crops would show a profound physiological stress response. Besides, we also investigated the effect of motorways on wood mice (Paper VI), predicting that those individuals living closer to the motorway would present higher physiological stress levels.

For each of the above mentioned papers we provide an abstract and introduction, describe material and methods used, finally presenting and discussing the results found. Later, in the concluding remarks section we summarize the major findings from the previous chapters also pointing out mitigation measures for the correct management and conservation of small mammal populations.

CHAPTER 1

RISK OF PREDATION



PAPER I

Navarro-Castilla, Á. & Barja, I. (2014). Antipredatory response and food intake in wood mice (*Apodemus sylvaticus*) under simulated predation risk by resident and novel carnivorous predators. *Ethology* 120: 90-98.

PAPER II

Navarro-Castilla, Á. & Barja, I. (2014). Does predation risk, through moon phase and predator cues, modulate food intake, antipredatory and physiological responses in wood mice (*Apodemus sylvaticus*)? *Behavioral Ecology and Sociobiology* 68: 1505-1512.

PAPER III

Navarro-Castilla, Á., Barja, I. & Díaz, M. (2015). Behavioral and physiological stress responses to predation risk by wild wood mice. *Journal of Mammalogy* (under review).

PAPER I

ANTIPREDATORY RESPONSE AND FOOD INTAKE IN WOOD MICE (*Apodemus sylvaticus*) UNDER SIMULATED PREDATION RISK BY RESIDENT AND NOVEL CARNIVOROUS PREDATORS

ABSTRACT

Chemical signals left by predators are a potential source of information about the risk of predation, and small mammals are known to take them into account when making decisions. We investigated whether wood mice (*Apodemus sylvaticus*) are more likely to avoid the faeces of resident predators (red fox *Vulpes vulpes* and common genet *Genetta genetta*) versus a novel predator (European pine marten *Martes martes*). Odour recognition would increase perceived predation risk and reduce food intake by individual mice. Wood mice response to predators was analyzed by live trapping using two untreated controls (baited/non baited) and traps experimentally manipulated with three predator treatments (faeces of red fox, common genet or pine marten). Traps were baited with 4 g of toasted corn and food intake by wood mice was determined as the amount of bait remaining in each trap. We found that traps treated with faeces of resident predators were the most avoided, and the number of captures in traps treated with pine marten faeces was similar to the control baited traps. The variation found in food intake was explained by the interaction between the types of treatment and breeding condition. Food intake was similar in control baited traps and in traps with faeces of pine marten, but when predation risk by resident predators (red fox and common genet) was simulated, breeders reduced food intake significantly as compared to non-breeders. These results indicate that predator recognition and feeding behaviour under predation risk depend on individual factors and the balance of costs-benefits in each particular predation risk situation at a given place and time.

Keywords: behavioural response, food intake, innate recognition, olfactory recognition, novel predators, resident predators

INTRODUCTION

In mammals, chemical signals are one of the main means of exchanging information, both within and between species (Gorman 1990). Most carnivores are territorial and they use different secretions from scent glands, urine and faeces to mark their territory (Hutchings & White 2000; Barja et al. 2005; Barja 2009). However, deposition of the scent marks on conspicuous places throughout their territory can also reveal their presence to prey species. Several field studies have shown that some small mammals are able to detect the presence of predators through chemical cues, in the urine and the faeces that predators use for territorial marking (Stoddart 1976; Dickman & Doncaster 1984; Gorman 1984; Barreto & Macdonald 1999; Fendt 2006) and the concentration of these cues may be used to assess predation risk (Lima 1998). This chemical recognition of predators could be a major advantage for small mammal species since in darkness, chemical signals may be especially important to detect predators and avoid a possible encounter. Therefore, animals with crepuscular and nocturnal patterns should be under strong selective pressure to detect and identify the chemical cues of predators (Müller-Schwarze 1999). Many studies have shown that avoiding areas marked by a predator is common in small mammals (Stoddart 1982; Dickman & Doncaster 1984; Calder & Gorman 1991; Dickman 1992). Interestingly, avoidance of a predators´ scent seems to depend on the species of predators. For example wood mice (*Apodemus sylvaticus*) avoided entering traps with red fox (*Vulpes vulpes*) faecal odour (Dickman & Doncaster 1984), yet not the faecal odour from stoat *(Mustela erminea)* or weasel *(Mustela nivalis)* (Stoddart 1976; Dickman & Doncaster 1984; Gorman 1984). Based on these different results, it is conceivable that prey species may behave differently based on different individual, environmental and evolutionary factors. When predators and prey have coexisted for long periods, prey often recognize and respond to predator cues by evolving behaviours or morphologies that reduce the chance of encounters with predators or enhance the chance of escape once detected (Lima & Dill 1990). In this regard, different studies have evaluated whether the detection and recognition of predators through chemical cues occurs without experience equally for all carnivore species (Calder & Gorman 1991; Kats & Dill 1998; Apfelbach et al. 2005; Fendt 2006). However, this may be conflicting when studying the response of native prey to introduced predators. Russell & Banks (2007) reported that some rodent species equally

avoid native versus introduced predator odours, whereas Mcevoy et al. (2008) found that the velvet-furred swamp rat (*Rattus lutreolus velutinus*) responded to a native predator scent but not to the odour of an introduced predator. So, if prey are unable to recognize the scent of novel predators, then this recognition failure could explain why some prey species are more vulnerable to newly introduced predators than to native ones (Salo et al. 2007). Dickman (1992) found that house mice (*Mus musculus*) avoided traps scented with the faecal odour of a novel predator, the quoll (*Dasyurus maculatus*), but the level of avoidance was weaker than for traps tainted with faecal odour of red fox and feral cat, which are natural predators of house mice. Furthermore, this avoidance response was not found in house mice living on islands where mammalian predators were absent, and if only cats were present, red fox and cat odours were avoid equally (Dickman 1992). This finding suggests that the predator responses to carnivore scent may be triggered by common volatile components in the faecal odours of some predators (Dickman & Doncaster 1984; Dickman 1992) and these compounds may be derived from a carnivore diet (Nolte et al. 1994; Kats & Dill 1998). Although there should be an advantage for prey able to detect predators through their chemical cues (Kats & Dill 1998), prey species might adjust this generalized response according to other components found in the odours of some predator species. So, Jedrzejewski et al. (1993) found that although bank voles (*Clethrionomys glareolus*) responded to the odour of six different mammalian predators, the responses to each one was different.

It is well known that perceived predation risk affects an animal´s decision-making process, affecting daily activity patterns (Fenn & Macdonald 1995; Díaz et al. 2005), feeding (Brown et al. 1998), use of space (Jacob & Brown 2000), reproduction (Lima 1998) and grouping behaviour (Barja & Rosellini 2008). Therefore, antipredatory response may be based on the skill of making behavioural decisions evaluating costs and benefits of each possible option (Lima & Dill 1990). For example, while seeking food, an animal itself risks becoming food, so, in response to predation risk, animals should balance food and safety (McNamara & Houston 1987; Lima & Dill 1990). Some studies have related the time used in food handling with vegetation cover as a predation risk factor (Lima & Valone 1986; Newman et al. 1988) but few studies have evaluated the amount of food eaten by prey under predation risk (Epple et al. 1993; Barreto & Macdonald 1999).

OBJECTIVES

The aim of this study was to test whether wood mice are able to recognize and avoid the faecal odour of three carnivores that feed on this species (red fox *Vulpes vulpes*, common genet *Genetta genetta* and European pine marten *Martes martes*). Red foxes and common genets are sympatric with wood mice, whereas European pine martens are not, and therefore, wood mice should be naïve to this predator. We hypothesize that wood mice will avoid the faecal odour of predators in the study area (red fox and common genet) but not the faeces from a novel predator (European pine marten). Since predation risk affects making decisions and may constrain foraging (Brown & Kotler 2004), we also analyzed food intake rates under simulated predation risk by these three carnivorous species and we expected that food intake would be lower when individuals were captured in traps treated with predator's faecal odours, and especially odours of sympatric predators.

MATERIAL AND METHODS

Study area

Fieldwork was conducted in a forest of central Spain at an altitude of 650 m a.s.l. within the Mediterranean climatic region. The study area covers approximately an extension of 259 ha consisting of dense forests of holm oak (*Quercus ilex ballota*) and pine reforestations (*Pinus pinea* and *Pinus pinaster*).

Live trapping and field data collection

Field experiments were conducted from April to October 2009. The response of wood mice to predators' faecal odour was examined through live-trapping in both types of habitats (holm oaks and pine forests). In each habitat, we selected two plots where 30 Shermann® live traps were placed in a 5x6 grid. Distance between traps was 7 m, they were oriented in the same direction and hidden under vegetation cover to protect animals from adverse weather conditions. Nest material (raw wool with natural lanolin) was used as bedding inside traps. All traps were baited with 4 g of toasted corn. Check-ups were done every 10-12 h, at dawn and dusk, to minimize the time that animals were kept and exposed to treatments. Traps were set on each habitat for five consecutive nights, being the total trapping effort of 2400 traps-night (30 traps per grid x 2 plots per habitat x 2 habitats x 5 nights x 4 trapping sessions). We used three predator treatments

(see details below) and two odourless control tests, one baited and other unbaited. Treatments were randomly interspersed in the grid, one per trap (Fig. 1), and during the consecutive five nights the location of the traps with the same treatment were kept the same. Predators´ presence was simulated by leaving an equal amount (5 g) of faeces at the entrance of some traps and faecal odours were renewed every day at dusk to prevent drying out rapidly what could decrease its effectiveness.

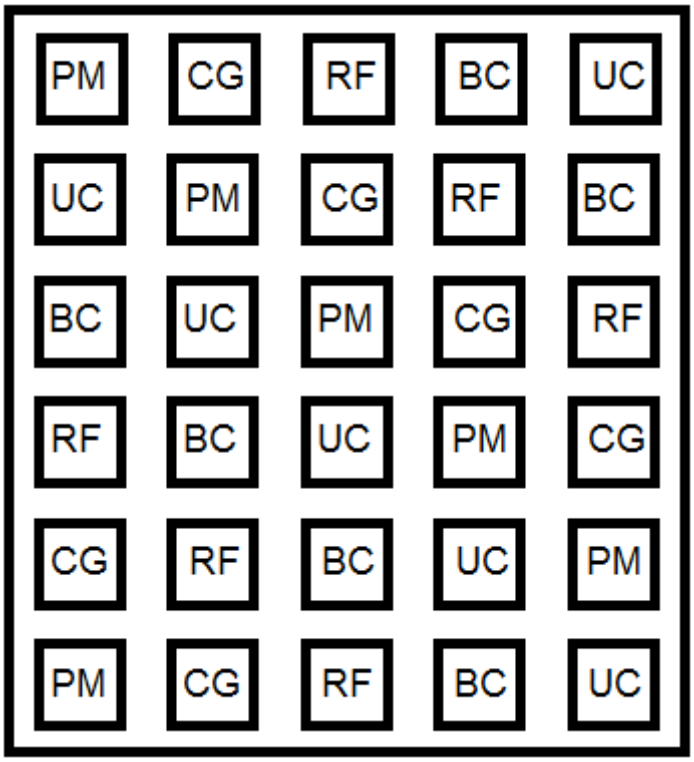


Figure 1. Distribution of the treatments in the grid. UC: unbaited control, BC: baited odourless control, PM: European pine marten faeces, RF: red fox faeces, GF: common genet faeces.

Each individual captured was checked for sex and breeding condition following Gurnell & Flowerdew (1994). Sex was determined using the anal-genital distance, which is smaller in females than in males. In breeding adult males the testicles enlarge quite markedly and usually descend into the scrotal sac whereas in breeding adult females the nipples on the abdomen and thorax are noticeable and the vaginal membrane appears perforated. Body weight was measured using a 100 g hand-held scale and age class estimated according to body weight: individuals weighing < 13 g were considered juveniles, from 13 g to < 20 g subadults and those weighing ≥ 20 g adults (Lewis 1968; Corbet & Harris 1991; Behnke et al. 1999). It has been reported

that in larger wild groups, higher density is usually related to generate a major degree of intraespecific competition, increasing aggression rates and social instability (Rogovin et al. 2003). Thus, we estimated wood mice abundance (considering only those individuals captured during live-trapping sessions excluding recaptured ones) to test any possible effect on decision making under predation risk.

All captured individuals were handled as fast as possible and they were released in the same place of capture. Captured animals were marked in non-conspicuous areas with harmless watery paints (red food colouring: Ponceau-4R E124) to identify possible recaptures. In this research, we fulfilled all the regulations concerning to handling and treatment of animals in accordance with the European Comumnities Council Directives of 24 November 1986 (86/609/EEC) for animal experiments and manipulations of animals were done under the permit of the Comunidad Autónoma de Madrid (Spain) reference number 10/422509.

Simulation of carnivores' presence

Since remains of wood mice are frequently found in the scats of predators like the common genet (Virgós et al. 1999), red fox (Padial et al. 2002), and European pine marten (Rosellini et al. 2008), predator treatments consisted in faecal odour of these predators. Faeces of red fox were collected from captive animals of the Cañada Real Open Center (Madrid, Spain), where animals are kept under semi-natural conditions for educational purposes. Common genet´s faeces were collected from latrines previously located in the study area and faeces of European pine martens were collected during surveying transects in the Natural Park Montes do Invernadeiro (NW Spain). Faeces from the three predator species were collected fresh, being considered as fresh when they presented a layer of mucus, a high level of hydration and strong odour (Liu et al. 2006; Barja et al. 2007). All faeces were frozen at - 20 ° C until used in the experiments. Although all faeces were freshly collected, volatile compounds vary in relation to seasonal or individual factors (Raymer et al. 1984; Andreolini et al. 1987; Jemiolo et al. 1991; Hayes et al. 2006b; Scordato et al. 2007). Thus, all collected faeces from the same species were thawed and mixed to obtain a homogeneous mixture, providing a similar degree of predation risk in all the treated traps, and therefore, we avoided possible bias in our results. Each predator treatment was made with 100 g of homogenized faecal

sample mixed with 100 ml of distilled water obtaining a mixture with a uniform and consisting similar texture as fresh faeces.

Food intake assessment

We used an electronic balance (Giros PG-500; precision 0.01 g) to weigh the remains of bait that had not been consumed by each individual captured to determine the amount of food eaten in each trap by each individual.

Statistical analyses

We analyzed capture data by means of the fit of log linear models to the six-way contingency table generated by the factors treatment, sex/age, breeding condition, abundance, habitat and presence/absence of capture. Structural zeros of breeding x juveniles were taken into account by joining sex and age of individuals as one variable. This statistical method is similar to an analysis of variance where total variance of data frequency is partitioned into different factors and factors interactions, permitting a test of significance of the different effects (Everitt 1977; Díaz et al. 1999). Chi-square (χ^2) tests were used to test the independence between the observed and expected frequencies of the different significant factors.

To analyze food intake under predation risk we used General Linear Model (GLM) using as response variable the amount of food taken. The response variable was transformed (log10 + 1) to fit normal distribution (Kolmogorov-Smirnov and Lilliefors tests) and homogenous variances criteria (Levene test). The independent variables were habitat (holm oak / pine forests), treatment (baited odourless control / red fox faecal odour / common genet faecal odour / European pine marten faecal odour), relative age (juveniles / sub-adults / adults), sex (males / females) and breeding condition (breeding / non-breeding) as fixed factors and wood mice abundance (log10 transformed) as the covariate.

In all statistical analysis we considered only those individuals captured during the live-trapping sessions excluding recaptured ones. Based on the number of captures we distinguished between a period of high abundance when the number of wood mice captured per trapping session was higher than 40 individuals (in April), and low abundance when the number of individuals captured was lesser than 20 (from June to October). Results were considered significant at $\alpha < 0.05$. Data are represented as mean

± standard error (SE). The software used to do statistical analysis was SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

The total number of wood mice captured during live-trapping, excluding recaptures, was 88 individuals. Results of the fit of log linear models showed that number of captures varied in relation to environmental and individual factors (Table 1). Number of captures was significantly larger during high abundance period ($P < 0.001$) and we captured more individuals in the holm oak than in the pine forest ($P < 0.001$). Adult males were captured more than adult females or juveniles ($P = 0.001$), and breeding individuals were captured less than non-breeding ones ($P < 0.001$). There were two significant two-ways interactions. Breeding individuals were captured more during the high abundance period, which coincided with the breeding season ($P < 0.001$). Adults were captured more in holm oak forest, whereas juveniles were captured more in the pine forests ($P < 0.001$). Wood mice were not captured equally in all traps and the number of captures varied depending on the treatment ($P = 0.033$; Fig. 2). The number of captures in unbaited odourless control traps was significantly lower than in the baited odourless control ones ($\chi^2 = 7.53$, df = 1, $P = 0.006$, n = 34). Traps with red fox faeces had few number of captures, followed by traps treated with faeces of common genet while in baited control traps we had a higher number of captures, however, these differences were significant only between the baited control traps and the red fox treated ones ($\chi^2 = 6.43$, df = 1, $P = 0.011$, n = 35). When predation risk was simulated with faeces of a non-resident predator, the European pine marten, individuals showed no avoidance, the number of captures in these traps was similar to the obtained in baited control traps ($\chi^2 = 0.08$, df = 1, $P = 0.78$, n = 52). Differences between captures in traps with resident predator faeces and in traps with non-resident predator faeces were statistically significant only between the red fox and the European pine marten treatments ($\chi^2 = 7.81$, df = 1, $P = 0.005$, n = 37). Interactions between treatment and the other studied factors were not statistically significant (Table 1).

Table 1. Results of the fit of log lineal model analyzing factors determining the capturability of wood mice.

Effect	df	G^2	*P*
Abundance level	1	114.75	0.000
Treatment	4	10.49	0.033
Sex/age	2	14.43	0.001
Breeding condition	1	26.22	0.000
Habitat	1	14.03	0.000
Abundance * Breeding condition	1	12.25	0.000
Sex/age * Habitat	2	20.37	0.000
Treatment x Abundance	8	3.58	0.893
Treatment x Sex/age	4	0.82	0.927
Treatment x Breeding condition	4	3.30	0.509
Treatment * Habitat	2	1.01	0.605

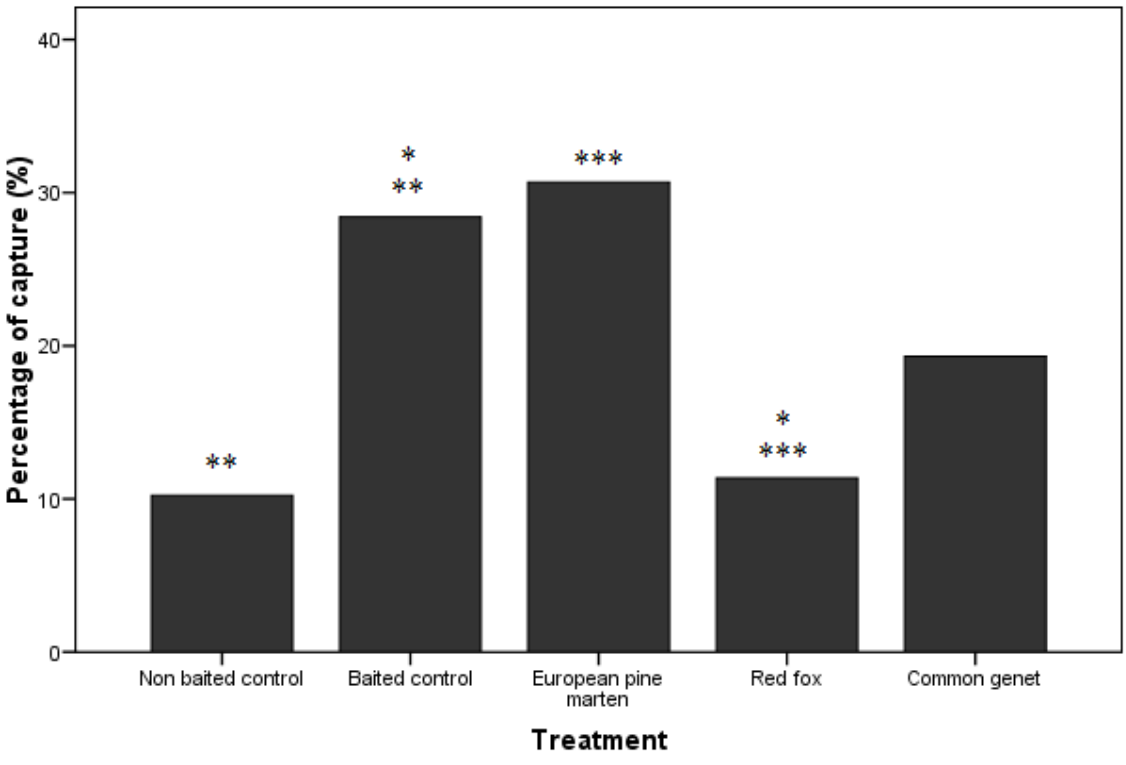


Figure 2. Percentage of wood mice captured in traps according to the type of treatment.

Food intake under predation risk

Results of the GLM for analyzing food intake are presented in Table 2. Although the amount of food consumed varied depending on the type of treatment (baited control:

2.20 ± 0.23 g, red fox faecal odour: 2.57 ± 0.46 g, common genet faecal odour: 2.60 ± 0.28 g, European pine marten odour: 2.42 ± 0.25 g) differences were not statistically significant (Table 2). In addition, there were some variation in food intake but differences were not significant for sex (Table 2; males: 2.28 ± 0.18 g, females: 2.66 ± 0.22 g), breeding condition (Table 2; non-breeding: 2.57 ± 0.16 g, breeding: 1.75 ± 0.18 g) and relative age (Table 2; juveniles: 2.49 ± 0.59 g, subadults: 2.65 ± 0.19 g, adults: 2.16 ± 0.20 g). Food intake did not vary with the abundance of wood mice nor with the type of habitat (Table 2; holm oak: 2.73 ± 0.35 g, pine reforestations: 2.32 ± 0.15 g). The interaction between treatment and breeding condition was statistically significant (Table 2). Whereas in baited control traps and in traps treated with European pine marten faeces breeders and non breeders consumed a similar amount of food, breeding individuals showed a lesser food intake when they were captured in traps treated with red fox or common genet faeces (Fig. 3).

Table 2. Results of the General Lineal Model testing the effects of environmental and individual factors on the amount of food intake by wood mice under predation risk.

Factor	**F**	**df**	***P***
Treatment	1.963	3	0.135
Sex	0.046	1	0.832
Breeding condition	0.205	1	0.653
Relative age	0.037	2	0.963
Abundance of wood mice	0.051	1	0.823
Habitat	0.593	1	0.446
Treatment * Habitat	0.169	1	0.683
Sex * Relative age	0.819	2	0.448
Sex * Breeding condition	0.029	1	0.866
Sex * Treatment	2.174	3	0.106
Sex * Habitat	0.378	1	0.542
Breeding condition * Treatment	3.256	3	0.031
Relative age * Breeding condition	0.409	1	0.526
Relative age * Treatment	1.088	4	0.375
Relative age * Habitat	0.397	2	0.675
Sex * Relative age * Treatment	0.345	2	0.711

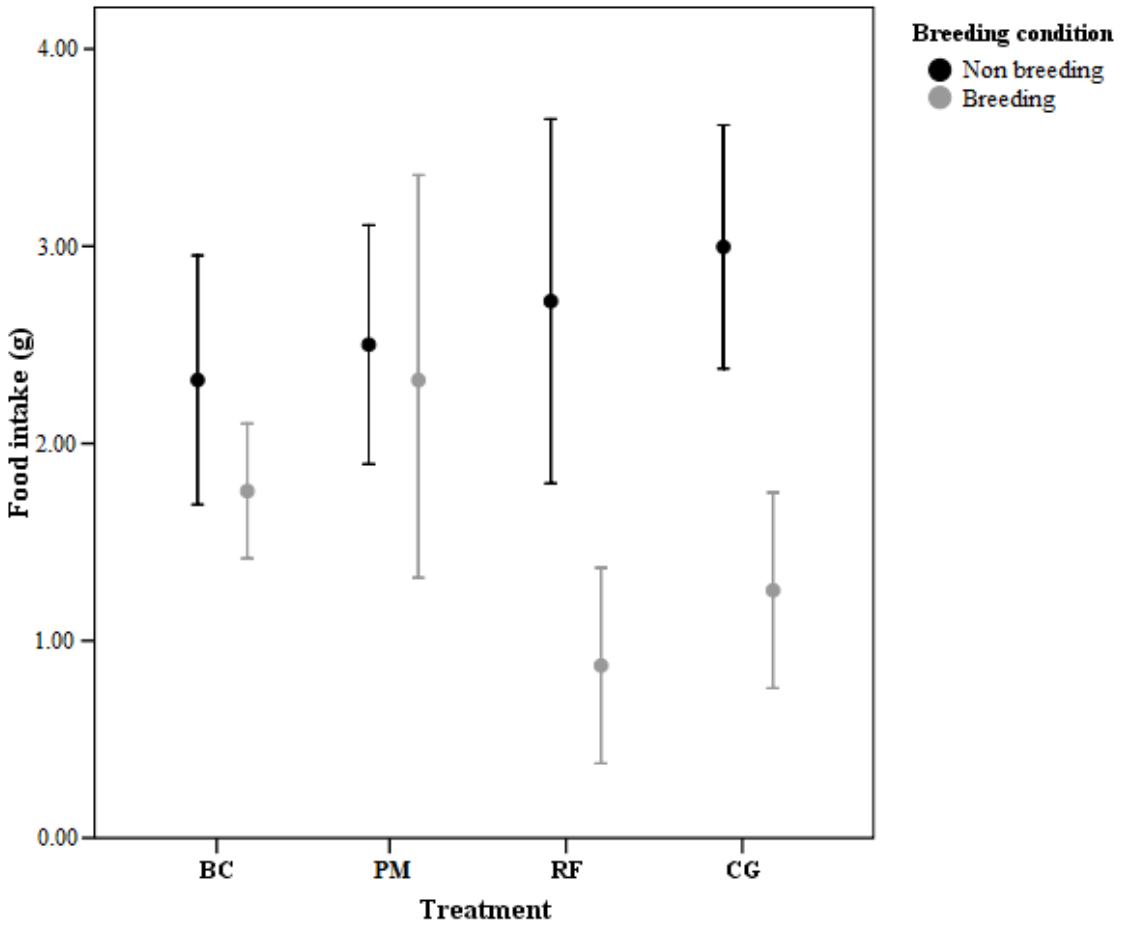


Figure 3. Comparison of the amount of food intake (g, mean ± SE) in relation to treatment and breeding condition. BC: baited odourless control, PM: European pine marten faeces, RF: red fox faeces, GF: common genet faeces.

DISCUSSION

There is huge evidence that small mammals consider predation risk when making decisions (e.g. foraging, feeding) (Lima & Dill 1990). Here the first decision is whether entering the trap or not. Individual mice clearly detect whether the trap has food or not (as demonstrated by the significant few number of captures in the unbaited controls), but mice cannot evaluate how much food there was inside. In our experiment, when mice found predator´s faeces in the entrance of a trap they had to choose between not entering or entering the trap to obtain food but risking predation. This decision could explain the low number of captures in traps treated with red fox faeces. However, there was not a total avoidance of these traps maybe because although chemical signals left by predators are an important source of information of predation risk in a certain place and at one particular moment, the risk of an encounter with a predator decreases as time

goes by, especially in the case of predators that have large territories (Kats & Dill 1998). In addition, faeces used to simulate the presence of predator got dry over time and the degree of risk decreased; thus, by decreasing the chance of being caught by a predator, the benefits of getting food could equal the costs of predation.

We also found that the avoidance of wood mice to traps treated with faeces from common genet was lower than those treated with red fox faeces. This result could be explained because wood mice show spatial and temporal changes in foraging behaviour in response to the presence of genets and although common genets prey on wood mice the encounters between predator and prey may be rare as showed (Díaz et al. 2005). In addition, it has been shown that in species where rate of encounter with predator is low, animals display a physiological response, which is not necessarily translated into a modification of behaviour (Eilam et al. 1999). Common genets defecate in latrines and therefore their faeces may be less indicative of their movement patterns compared with those predators whose faeces are scattered throughout their territory, as in the case of the red fox (Dickman 1992; Hayes et al. 2006a). Thus, avoidance of places with faecal odour of common genets may not significantly reduce their chances of being caught by this predator, what means that avoidance might not lead to large benefits relative to costs which would result in failure to obtain food. Díaz et al. (2005) suggested that wood mice would rather use auditory cues to detect genets since responses to their presence were quite fast. So, the results of our experiments could suggest a system of predator-prey coevolution. Common genets concentrate their faeces in latrines failing to report their movement patterns and wood mice do not take the information given by predator marking behaviour as a real cue of potential predation risk. This response can have evolved or have been simply learned.

Although the European pine marten is a potential predator of wood mice, our results surprisingly showed that wood mice did not avoid traps treated with marten faeces. Faeces from European pine marten are clearly indicative of their movements, because their faecal marks are deposited in all its territory (Hutchings & White 2000; Barja 2005; Barja et al. 2011). In view of these results it could be that the ability of prey to detect and avoid predators depends in part on the experience, the ecology and the evolutionary history between predator and prey. Thus, the process of decision making under predation risk turn problematic when prey faces up to introduced predators (Jones

et al. 2004; Russell & Banks 2005). Wood mice could be better prepared to recognize predator species living nearby than predator species introduced recently or allopatric. Native species may be less able to respond to predation risk when it comes from an introduced or a novel predator due to a discordant historical evolution between both species (Banks 1998; Banks 1999; Blumstein et al. 2002; Russell & Banks 2005), however, over time wood mice could be able to recognize and avoid European pine marten faecal odour as it has been shown in other prey species when new predators were introduced (Russell & Banks 2007).

Food intake under predation risk

The variation in the amount of food ingested by wood mice during the time that individuals were kept into traps was explained by the interactions of type of treatment x breeding condition. According to the risk allocation hypothesis (Lima & Bednekoff 1999), prey should allocate more feeding effort in low risk situations and they trade-off its feeding effort and other activities to optimise the energy spent on more antipredator behaviour in high risk situations. Although experiments testing this idea have yielded mixed results (Hamilton & Heithaus 2001; Sih & McCarthy 2002; Van Buskirk et al. 2002; Pecor & Hazlett 2003; Sundell et al. 2004), some studies have shown that when animals are under predation risk they decrease the time to handle the food (Lima & Valone 1986; Newman et al. 1988) and even reduce food intake (Epple et al. 1993; Barreto & Macdonald 1999). We found this behaviour in breeding individuals who really decreased their food intake when they were captured in traps treated with faeces of resident predators (red fox and common genet), however, non-breeders showed a non-significant increase in food intake when they were trapped in traps with resident predator faeces. This difference could be in part due to the breeding condition, breeders could be more careful under risky situations avoiding feeding and giving priority to survival for matching and breeding. We could not control the time that each animal spent inside traps but if the non breeders would had pass more time inside traps than the breeders did, these differences could be explained by Lima & Bednekof (1999) who proposed that if the period of high risk last long enough the animal is forced to decrease its antipredatory behaviour and forage also during this high risk situation to meet its energy demands.

Finally, we can conclude that wood mice seem to recognize and avoid the odour of carnivore resident predators, the red fox and the common genet. This ability seems to be modulated by the assessment of costs-benefits in each moment and by each individual. For instance, an encounter with the odour of a predator may indicate that it is still around and more likely to be encountered, or it may provide no information of the probability of re-encountering the predator again if the predator moves through the environment haphazardly. Thus, the odours of predators are chemical relevant compounds for prey what means an increase in predation risk if they stay in the odour´s closeness (Gurney et al. 1999), but the olfactory research behaviour is an important component of the process of assessment predation risk (Blanchard et al. 1989). Thus, chemical signals are complex mixtures, not only chemical, and responses to them are not stereotypical or generalized, but that will depend on context, past experience and the status of each individual (Gorman & Trowbridge 1989).

PAPER II

DOES PREDATION RISK, THROUGH MOON PHASE AND PREDATOR CUES, MODULATE FOOD INTAKE, ANTIPREDATORY AND PHYSIOLOGICAL RESPONSES IN WOOD MICE (*Apodemus sylvaticus*)?

ABSTRACT

Predation influences the ecology and behavior of prey species and it is well known that the risk of predation affects prey's decision making. We investigated whether predation risk through moon phase and exposure to the faecal odour of a natural predator, the red fox *Vulpes vulpes,* affect feeding behaviour and physiological response in wood mice (*Apodemus sylvaticus*). Antipredatory response was studied by live trapping under new and full moon in odourless control areas and areas experimentally manipulated with red fox fresh faeces. Food intake by individuals was determined as the amount of bait remaining in each trap and the physiological response was measured non-invasively analyzing faecal corticosterone metabolites (FCM). Traps treated with faeces of red fox were the most avoided, and this avoidance was more significant during full moon. Food intake by wood mice varied according to the moon phase being significantly lower under full moon nights. We found sex, breeding condition and weight of individuals explaining the variation found in FCM concentrations, but no changes in FCM levels due to moon phase or exposure to red fox faeces were detected. These results indicate that wood mice avoid red fox faecal odour and, this antipredatory response as well as feeding behavior are significantly influenced by moon phase. However, no physiological response was found due to predation risk suggesting that wood mice do not take these predation cues enough reliable to experience physiological changes.

Keywords: behavioural response, faecal corticosterone metabolites, faecal predator cues, feeding behaviour, lunar cycle, olfactory recognition, predator avoidance

INTRODUCTION

In nature, animals are exposed to a wide range of threats and dangers with predators, therefore, predation strongly influences the ecology of prey species both directly via removal of prey and indirectly via predator´s effects on prey behavior (e.g., Abrahams & Dill 1989; Lima & Dill 1990; Kats & Dill 1998; Apfelbach et al. 2005; Navarro-Castilla & Barja 2014a). Since predation represents an important cause of death for many small mammals it has led to a variety of predator detection mechanisms and antipredatory responses by prey species (Lima 1998).

Small rodents depend on the detection of a predator prior to actual contact, thus, it is crucial for an animal to recognize and respond adaptively to its predators. Most carnivores use different secretions from scent glands, urine and faeces to mark their territory (Hutchings & White 2000; Barja et al. 2005; Barja 2009; Piñeiro et al. 2012), and several studies have shown that small mammals are sensitive to the scent of potential predators avoiding such odours without needing other cues (Stoddart 1982; Dickman & Doncaster 1984; Calder & Gorman 1991; Jedrzejewski et al. 1993; Navarro-Castilla & Barja 2014a).

Recognition and avoidance of predators may be really important for small mammals since while they are doing daily activities, such as foraging or feeding, they are exposing themselves to predators. Therefore, predator cues are known to affect animal´s decisions, and so, prey species often alter their behaviour in response to the auditory, visual and chemosensory cues of their predators (Lima & Dill 1990; Kats & Dill 1998). In addition, environmental factors (e.g. moonlight and habitat complexity) are important sources of information influencing predation risk perception for prey species. For example, moonlight has been shown to make organisms more conspicuous or detectable to their predators (Kotler et al. 1991; Longland & Price 1991). The increased risk of predation with moonlight makes rodents to avoid illuminated nights and open areas, favoring less activity on full moon nights and microhabitat preference for sheltered environments (Kaufman & Kaufman 1982; Wolfe & Summerlin 1989; Díaz 1992; Eilam 2004; Kotler et al. 2010; Penteriani et al. 2013). These behavioral changes under perceived predation risk situations probably reduce the probability of an encounter with a predator, but they involve inevitable trade-offs and costs between the benefits of safety avoiding predation and the costs of missing feeding or reproduction

(Abrams 1986; Lima & Dill 1990; Brown et al. 1999; Brown & Kotler 2004; Penteriani et al. 2013). Thus, antipredator behavioral responses would be expected to be used only when an animal has an accurate assessment of the current predation risk and making decisions choosing those behavioral options which maximizes their fitness.

However, the perception of predators as a threat should not be restricted to behavioural responses because, under certain situations, some prey species display a physiological response which is not translated into a modification of behavior (Eilam et al. 1999). Besides, predators can induce physiological responses in their prey not only by physically attacking them but also by making them fearful of an imminent potential attack (Boonstra et al. 1998; Hirschenhauser et al. 2000; Korte 2001). Measuring glucocorticoid levels to evaluate physiological responses during stressful situations has been showed as a useful technique (Möstl & Palme 2002; Touma & Palme 2005; Sheriff et al. 2011; Barja et al. 2012; Navarro-Castilla et al. 2014ab). However, physiological responses of prey species subjected to predation risk have yield mixed results depending on the species studied. While in some species increased physiological responses due to predation risk were found (e.g. snowshoe hare: Boonstra et al. 1998; wild rabbit: Monclús et al. 2009; voles and mice: Eilam et al. 1999, this was not the case in others (voles: Fletcher & Boonstra 2006; Ylönen et al. 2006).

OBJECTIVES

The present study was undertaken to test if there was any change in wood mice (*Apodemus sylvaticus*) behavior and physiological stress response with the exposure to increased predation risk by predator odour. In addition, we studied whether the reactions to predator odour were mediated by moon phase. Thus, we studied over the course of full and new moon phases and under threat of red fox (*Vulpes vulpes)* how predation risk affected predator avoidance, food intake and the physiological stress response in wood mice. Red fox predation is a significant threat for small mammals, especially rodents which are a major food source for this species (Padial et al. 2002). Fox odour has been used previously to simulate predation risk in field studies (Dickman & Doncaster 1984; Navarro-Castilla & Barja 2014a). Thus, we predicted wood mice avoiding areas marked with red fox faeces, especially during full moon nights. We also expected changes in feeding behavior and physiological stress responses occurring under the increased predation risk. Due to an increase in predation risk, we predicted a

decrease in food intake: (1) under full moon; and (2) during exposure to red fox faeces. In addition, we expected the exposure to red fox faecal odour to evoke physiological stress responses in wood mice, and we also expected physiological stress responses increasing during full moon.

MATERIAL AND METHODS

Study area

Fieldwork was carried out in a Mediterranean forest of central Spain at an altitude of 650 m a.s.l. within the Mediterranean climatic region. The study area covers approximately an extension of 259 ha constituted mainly by dense forests of holm oak (*Quercus ilex ballota*) and interspersed pine reforestations (*Pinus pinea* and *Pinus pinaster*).

Live trapping and data collection

The response of wood mice to predator's faecal odour was examined through live-trapping. Field experiments were conducted in two holm oak areas. In each area, we selected two separated plots where 15 Shermann® live traps were placed in a 3x5 grid, they were oriented in the same direction and with 7 m of separation between traps. Traps were hidden under vegetation cover to protect animals from adverse weather conditions, and nest material (raw wool) was provided inside as bedding. Check-ups were done twice-daily, at dawn and dusk, to minimize the time that animals were kept. All traps were baited with 4 g of toasted corn. Trapping sessions were done during the days close to new and full moon phases. Since trapping sessions lasted 5 days, we checked for the brightness day during full moon and the darkness one during new moon and trapping was started 2 days before the main day and finished two days after. Live trapping was carried out in November and April when wood mice densities are high in the study area (Navarro-Castilla & Barja 2014a). Traps were set on the field during five consecutive nights, being the total trapping effort of 1200 traps-night (30 traps per grid x 2 plots x 2 trapping sessions x 5 nights x 2 lunar phases).

Red fox avoidance by wood mice has been previously shown in Navarro-Castilla and Barja (2014a), so, we used red fox faeces as predator treatment. In each area, we assigned a predator faeces treatment for one plot and the other plot was set as odourless control test. The location of traps and each treatment in the plots were kept the same

during all the study. Red fox´s presence was simulated by leaving an equal amount (5 g) of faeces at the entrance of the traps and faecal odour was renewed every day at dusk to prevent drying out rapidly what could decrease its effectiveness.

We followed Gurnell & Flowerdew (1994) to determine the sex and breeding condition of each individual captured. Sex was determined using the anal-genital distance, which is longer in males than in females. In breeding adult females, the nipples on the thorax and abdomen are noticeable and the vaginal membrane appears perforated while in breeding adult males the testicles enlarge quite markedly and usually descend into the scrotal sac. Body weight of individuals was measured with a 100 g hand-held scale. All captured animals were marked in non-conspicuous areas with harmless watery paints (red food colouring: Ponceau-4R E124) to identify recaptures. Individuals were handled as fast as possible and they were released at the same point of capture.

Simulation of predator´s presence

The predator treatment consisted of red fox faeces. Faeces were collected from captive animals of the Cañada Real Open Center (Madrid, Spain). All faeces were fresh collected, i.e., faeces presented a layer of mucus, a high level of hydration and strong odour (Liu et al. 2006; Barja et al. 2007). All collected faeces were frozen at - 20 ° C until used in the experiments. Since volatile compounds vary in relation to individual or seasonal factors (Raymer et al. 1984; Andreolini et al. 1987; Jemiolo et al. 1991; Hayes et al. 2006b; Scordato et al. 2007; Martín et al. 2010), for the experiments, all red fox collected faeces were thawed and mixed to obtain a homogeneous mixture. Thus, we provided a similar degree of predation risk in all the treated traps, and therefore, we avoided possible bias in our results. Predator treatment was made with 100 g of homogenized red fox faecal sample plus 100 ml of distilled water, obtaining a mixture with a uniform and consisting similar texture as red fox fresh faeces.

Food intake assessment

The remains of bait not consumed by each individual captured were weighed with an electronic balance (Giros PG-500; precision 0.01 g) and used to determine the amount of food eaten in each trap by each individual.

Faeces collection and quantification of faecal corticosterone metabolites (FCM)

Faeces were collected from each trap where an individual was captured. Faeces were collected at sunrise to avoid the influence of a circadian rhythm in excretion patterns (Touma et al. 2003; Touma et al. 2004). Only fresh faecal samples were collected to prevent the action of environmental conditions and degradation by microorganisms (Millspaugh & Washburn 2003; Möstl et al. 2005; Barja et al. 2012). Traps where urine was detected were rejected to avoid cross contamination of faecal samples. Since peak FCM concentrations appear in the faeces about 10 h after the injection of adrenocorticotropic hormone (ACTH) in mice (median: 10 h, range: 8–12h; Touma et al. 2004), traps were reviewed within 10 hours to avoid any possible effect of the capture itself on FCM levels. Faecal samples were frozen at -20º C until analysis.

FCM were extracted from the faeces according to the previously described methods of Touma et al. (2003) and Palme et al. (2013). Briefly, faeces were dried until constant weight and each faecal sample was homogenized and 0.05 g were weighed and mixed with 1 ml of 80% methanol in an eppendorf tube. Eppendorf tubes were shaken for 30 min on a multivortex and then centrifuged 15 min at 2500 *g*. The obtained supernatants were diluted 1:10 with assay buffer and stored at -20ºC until analysis. To analyze FCM we used an already established 5α-pregnane-3ß,11ß,21-triol-20-one enzyme immunoassay (EIA) measuring metabolites with a 5α-3ß,11ß diol structure, which has been used previously in this species and in laboratory mice (Touma et al. 2003; Touma et al. 2004; Navarro-Castilla et al. 2014b).

Statistical analysis

We used Chi-square (χ^2) tests to check the independence in the number of captures between the observed and expected frequencies of the different predation risk studied factors. Food intake under simulated predation risk was analyzed by General Linear Models (GLMs). The response variable was the amount of food taken and the independent variables were season (autumn / spring), moon phase (new moon / full moon), treatment (odourless control / red fox faecal odour), sex (males / females), breeding condition (breeding / non-breeding) and recapture as fixed factors, and weight of individuals as covariable. Finally, we used GLMs to analyze factors explaining variation in FCM. Independent variables were season, moon phase, treatment, sex,

breeding condition and recapture set as fixed factors and body weight of individuals as continuous covariate.

Continuous variables were previously checked to fit normal distribution (Kolmogorov-Smirnov test) and homogenous variances criteria (Levene test), and log transformed when it was necessary to fit both criteria. Results were considered significant at $\alpha < 0.05$. Data are represented as mean ± standard error (SE). The software used to do statistical analysis was SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

We had a total of 93 wood mice captures during live-trapping. The number of individuals captured depended on the type of treatment, being significantly lower in red fox treatment traps than in the control test ones (38.1 % vs. 61.9 %, respectively) (χ^2= 7.84, df = 1, P = 0.005; N = 93). This pattern was similar for new individuals (χ^2= 3.57, df = 1, P = 0.059, N = 63) and for the recaptured ones (χ^2= 4.80, df = 1, P = 0.028, N = 30; Fig. 4). Red fox avoidance was noticeable during both moon phases, however, differences between the number of captures in relation to treatment were more accurate and significant during full moon phase (χ^2 = 5.12, df = 1, P = 0.024) than under new moon (χ^2 = 2.81, df = 1, P = 0.093; Fig. 5).

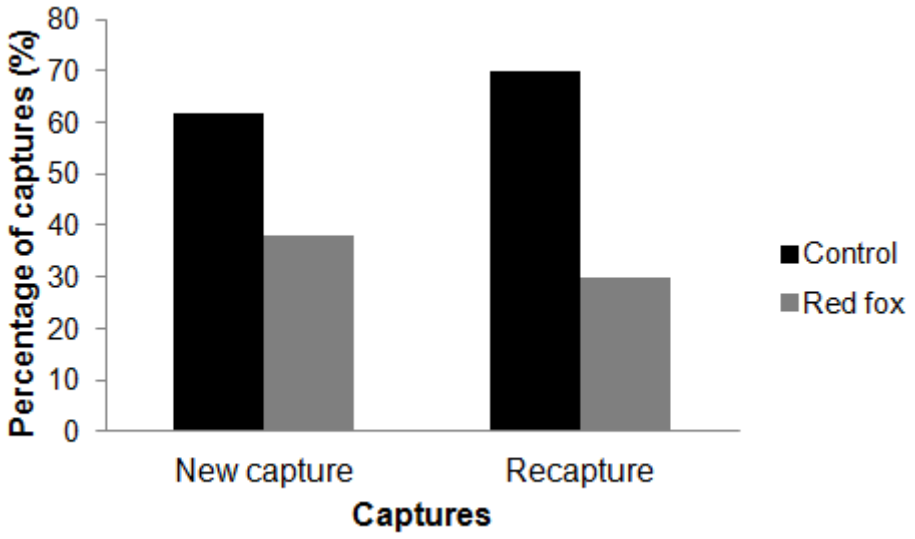


Figure 4. Percentage of wood mice captured and recaptured in traps according to the type of treatment.

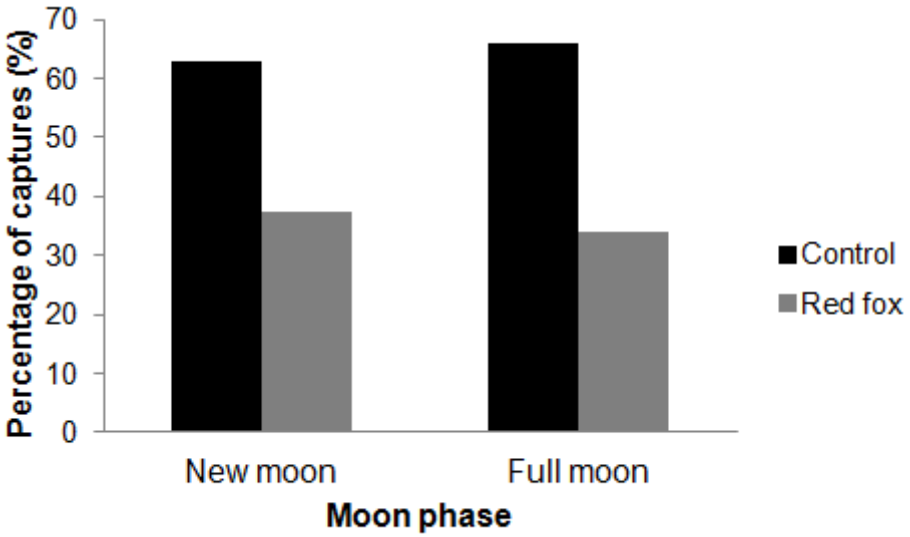


Figure 5. Percentage of wood mice captured in each treatment in relation to full and new moon phases.

Food intake

Results of the GLM analyzing food intake are presented in Table 3. The only factor influencing food intake by wood mice was the moon phase ($F_{1,93} = 6.758$, $P = 0.012$). Individuals consumed more food during the new moon (3.17 ± 0.17 g) whereas under full moon they decreased food intake (2.22 ± 0.14 g) (Fig. 6). Interactions between factors were not statistically significant.

Table 3. Effects of environmental and individual factors on the amount of food intake in wood mice under predation risk.

Factor	F	df	*P*
Intercept	2.083	1	0.154
Moon phase	6.758	1	0.012
Treatment	0.107	1	0.744
Season	2.458	1	0.122
Recapture	0.90	1	0.765
Sex	0.702	1	0.405
Breeding condition	1.266	1	0.265
Weight	1.343	1	0.251

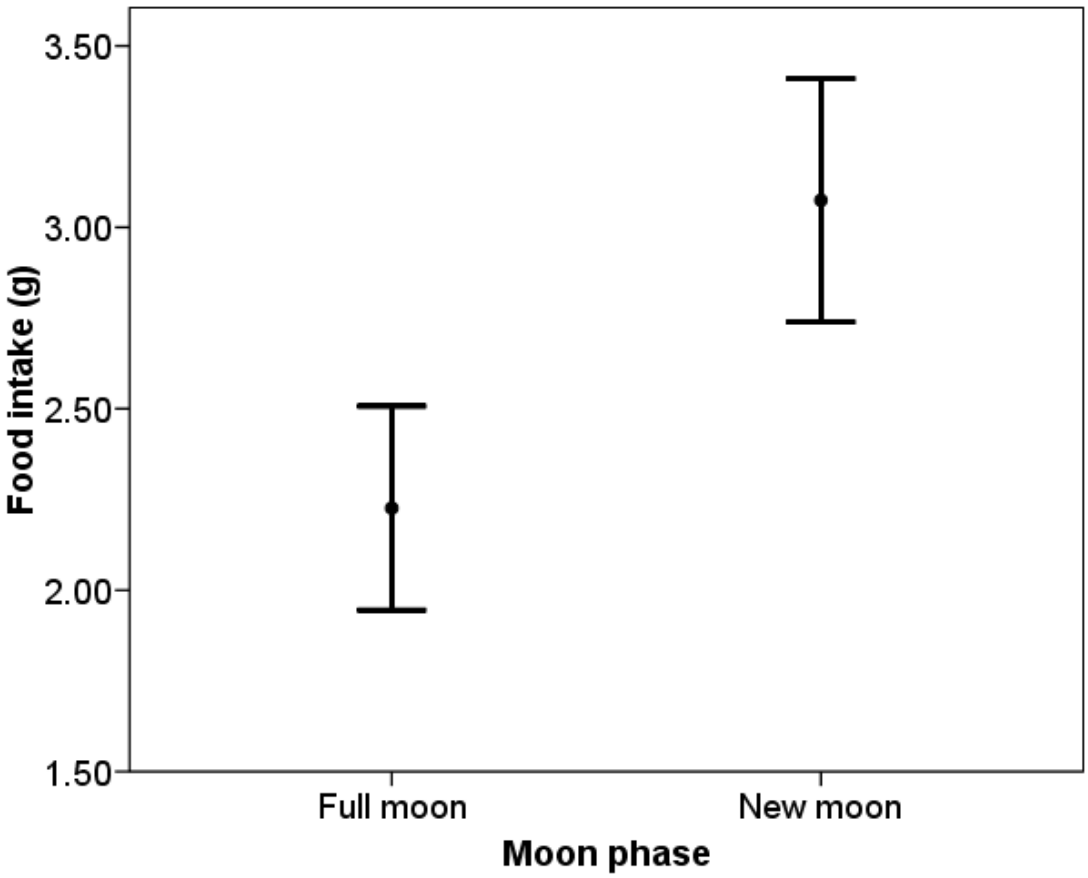


Figure 6. Food intake by wood mice (g, mean ± SE) during full and new moon.

Faecal corticosterone metabolites (FCM)

The statistical model (Table 4) showed that significant factors explaining the variation found in FCM levels were sex, breeding condition and body weight of individuals. Levels of FCM differed between sexes, concentrations of FCM were lower in males (3142 ± 635 ng/g dry faeces) than in females (4846 ± 1030 ng/g dry faeces) ($F_{1,93}$ = 8.07, P = 0.006). Breeding individuals showed higher FCM levels (8080 ± 1487 ng/g dry faeces) compared to the non-breeding ones (1784 ± 240 ng/g dry faeces) ($F_{1,93}$ = 4.08, P = 0.049). FCM levels correlated positively with body weight of animals ($F_{1,93}$ = 5.86, P = 0.019). However, other factors or their interactions were not statistically significant.

Table 4. Effects of environmental and individual factors on the amount of food intake on faecal corticosterone metabolites in wood mice under predation risk.

Factor	F	df	*P*
Intercept	128.796	1	0.000
Moon phase	0.307	1	0.582
Treatment	1.327	3	0.275
Season	1.862	1	0.653
Recapture	0.237	1	0.628
Sex	8.068	1	0.006
Breeding condition	4.077	1	0.049
Weight	5.861	1	0.019

DISCUSSION

The present study showed that wood mice capturability was significantly lower in traps with red fox faeces. This result is consistent with previous works where wood mice avoided red fox faecal odour (Dickman & Doncaster 1984; Navarro-Castilla et al. 2014a) and it extends the previous findings by showing that this predator avoidance was clearly influenced by moon light. We found that the difference in the number of captures in red fox treatment traps compared to the control ones was significantly lower during the full moon. This could be explained because small mammals perceived illumination as an increase in predation risk (Kaufman & Kaufman 1982; Daly et al. 1992; Eilam 2004; Kotler et al. 2010). These results suggests that red fox faeces and moon light are clearly synergic factors influencing predation risk perception in wood mice since foraging under these conditions is risky by increasing the possibility of being detected by a predator. But the antipredatory strategies are costly, maybe this is the reason why there was not a total avoidance of red fox treated traps. So, individuals seem to modulate their antipredator responses to the risk of predation perceived in each particular situation when making decisions (Lima & Dill 1990).

Food intake

Generally, prey spend more feeding effort under low risk situations and they give up foraging, feeding and other daily activities to antipredatory behavior when they are under high risk situations (Lima & Bednekoff 1999). Sherman traps could have acted as refuge for wood mice, however we found moon light influencing negatively food

intake. Individuals could perceived the risk of predation while foraging, and once inside the trap, moon light could perfectly go inside through several trap´s grooves. Thus, individuals seem to perceive full moon as an increase in predation risk even when they are inside the trap, and wood mice displayed a reduction in food intake during full moon compared to new moon phase. This result is in accordance with other studies where animals decreased foraging activity, handling or food intake when they were under predation risk situations (Lima & Valone 1986; Newman et al. 1988; Díaz 1992; Epple et al. 1993; Barreto & Macdonald 1999; Kotler et al. 2010). However, although predator faeces were previously found to have an effect on food intake in the wood mouse (Navarro-Castilla & Barja 2014), in this field experiment the exposure to predator cues did not have any effect on feeding behavior as similarly found (Sundell et al. 2004). It could be that after a long-lasting faecal cue without a real attack of a predator, individuals might have habituated to the red fox odour and they did not alter food intake in response. Alternatively, according to Lima & Bednekoff (1999) and Bednekoff & Lima (2004) individuals under a long-lasting high risk situation are forced to decrease their antipredatory behavior and forage to compensate energy demands. A possible explanation for the observed moon phase but not predator odour effect on food intake could be that indirect cues could be more important factors in the assessment of predation risk than direct cues from predators (Orrock et al. 2004). However, this would not occur in all scenarios since Díaz et al. (2005) found that wood mice reduced foraging activity in those areas where common genet activity was registered, but this antipredatory response was independent of the moon phase. Therefore, it seems that moonlight and predators influence animal´s decision, but responses seemed to be modulated by the different predation cues and the balance of costs-benefits in each predation risk situation.

Physiological stress response

Environmental and disturbing factors can act as potential stressors for natural populations causing physiological changes (Wingfield et al. 1997), however, in our experiment, significant differences found in faecal corticosterone metabolites (FCM) levels were explained by sex, breeding condition and weight of individuals. Females showed higher levels than males which is in accordance with other studies done in this and other rodent species (Touma et al. 2004; Navarro-Castilla et al. 2014ab).

Differences found in FCM levels could be partly due to differences in the metabolism of glucocorticoids between both sexes (Touma et al. 2003). Several studies have reported higher glucocorticoid concentrations during breeding (Dantzer et al. 2010; Navarro-Castilla et al. 2014a), and, as previously showed Navarro-Castilla et al. (2014b) for this species, we found breeding individuals showing higher FCM levels. Because of the request for maternal investment during pregnancy and lactation, breeding females experience several metabolic changes (Bauman 2000; Strier et al. 2003; Reeder & Kramer 2005) where glucocorticoids play a major role (Tataranni et al. 1996). The strong competition among males for mating females could generate an increase in aggressive behavior what might explain the high FCM levels for breeding males. Body weight of individuals was positively correlated with FCM levels, coincident with previous results for this species (Navarro-Castilla et al. 2014). Since weight of wood mice is closely related with the age of individuals (Gurnell & Flowerdew 1994), our results suggest young individuals showing lower physiological stress reactions than adults. Thus, individuals promote behavioral and physiological responses to cope with stressors that could differ by age-related alterations (Hauger et al. 1994).

Physiological responses due to predation risk were previously found for different mammal species including rodents (Boonstra et al. 1998; Eilam et al. 1999; Monclús et al. 2006; Monclús et al. 2009). However, in our study FCM levels did not vary with moon phases nor after following exposure to red fox odour. This lack of influence of predation risk factors on the physiological stress response parallels with results found by Ylönen et al. (2006) and Fletcher & Boonstra (2006) since they did not found weasel odour having any physiological effect in different vole species. A likely explanation for our results is that wood mice did not experience a physiological response to predator odour because odour in the absence of other stimuli could not be a reliable indicator of immediate predation risk (Orrock et al. 2004). Alternatively, prey species could not experience physiological stress responses each time they are exposed to predation risk since they would probably suffer chronic stress which would negatively affect their fitness (Möstl & Palme 2002).

In conclusion, wood mice recognized and avoided red fox faecal odour and this antipredatory response was higher under full moon nights due to a higher perceived risk of predation. Our research also suggests that wood mice use indirect cues of predation

risk, moonlight, rather than the predator faecal cues to assess the risk of predation before altering feeding behaviour. Neither moonlight nor predator cues triggered physiological stress responses maybe because these cues were not reliable enough to warrant the costs associated with a physiological change. However, antipredatory behavior of prey is likely to be affected by its previous experience with predation risk (Lima & Bednekoff 1999). Thus, individual variability and noncontrolled stressors, due to the experimentally outdoor conditions, could have influenced our results. Therefore, further research would be necessary to better understand the effect of different predation risk factors on the behavioural and physiological responses of wood mice.

PAPER III

BEHAVIORAL AND PHYSIOLOGICAL STRESS RESPONSES TO PREDATION RISK BY WILD WOOD MICE

ABSTRACT

Threat of predation is key to understand the ecology and behaviour of prey since they face the risk of being eaten by predators daily. We studied wood mice *Apodemus sylvaticus* behavioural and physiological responses to simulated predation risk. Risk avoidance in relation to indirect and direct cues was analyzed by live trapping unde new and simulated full moon conditions, and pairing sites with odourless control traps and traps treated with fresh faeces of common genet *Genetta genetta*. Foraging behavior was analyzed by video recording mice activity around traps. All traps were supplied with 4 g of roasted corn and food intake was calculated as the amount of bait remaining in each trap. Finally, we measured faecal corticosterone metabolites (FCM) as indicators of physiological stress response. Wood mice were less captured during full moon, whereas only non-breeding adult males clearly avoided common genet traps. Foraging activity was lower when individuals faced common genet faeces. Food intake only varied in relation to breeding condition, with breeding individuals showing the lowest food intake. FCM levels varied according to sex, breeding condition and body mass, but neither moonlight nor predator odour influenced them. Our results suggest that both direct and indirect cues of predation risk affected wood mice behaviour, although behavioural responses seem to be modulated by differential costs-benefit balances related to the individual's sex, age and breeding status. Lack of physiological responses to predation risk cues suggests that these cues were not reliable enough to outweigh the energetic costs associated to such physiological responses.

Keywords: common genet, faecal predator cues, feeding, foraging, moon phase, predator avoidance

INTRODUCTION

By definition, predatory interactions reduce the fitness of prey to zero after the interaction (Lafferty & Kuris 2002). Thus, there is a strong selective pressure in prey species for developing strategies aimed to avoid these interactions (Lima & Dill 1990). Prey respond to cues of the risk of being predated in a number of behavioural and physiological ways by using both indirect cues (dangerous habitats or environmental conditions) and direct cues (signals associated to the presence of predators such as urine, faeces or sounds; Orrock et al. 2004). Predation represents one of the most important causes of death for small mammals and it strongly influences prey ecology directly by killing (Brown et al. 1999; Hanski et al. 2001) or through indirect effects on prey behavioral responses to predators (Lima & Dill 1990; Apfelbach et al. 2005; Díaz et al. 2005; Navarro-Castilla & Barja 2014ab). Since animals are exposed to a huge range of dangers and treats by predators, they have developed a variety of predator detection mechanisms and antipredatory responses to minimize or avoid predation risk (Lima & Dill 1990; Kats & Dill 1998; Lima 1998).

Since most carnivores use secretions from glands, urine and faeces to mark their territory (Hutchings & White 2000; Barja & List 2006; Barja 2009; Martín et al. 2010; Piñeiro et al. 2012), different studies have shown several rodent species being sensitive to the scent of potential predators avoiding such chemical signals without needing other cues (Stoddart 1982; Dickman & Doncaster 1984; Calder & Gorman 1991; Jedrzejewski et al. 1993; Navarro-Castilla & Barja 2014ab). Furthermore, prey species often alter their behavior in response to the auditory, visual and chemosensory cues from predators (Lima & Dill 1990; Kats & Dill 1998). Thus, since prey species risk themselves while doing daily activities, there are tradeoffs between antipredator behavior and other fundamental activities like foraging or feeding (Sih 1980; Brown 1988; Brown et al. 1988; Orrock et al. 2004). In addition, antipredatory behavior can be strongly influenced by the environment. Thus, an increase in predation risk perception through lower habitat complexity or higher moon light illumination has show rodent species avoiding open areas and decreasing activity during full moon nights (Kaufman & Kaufman 1982; Kotler et al. 1988; Wolfe & Summerlin 1989; Kotler et al. 1994; Brown et al. 2001; Kotler et al. 2002; Eilam 2004; Kotler et al. 2010).

However, predator's threat should not be restricted only to behavioral responses because, under certain risky situations, prey may also display physiological responses which are not translated into a modification of behaviour (Eilam et al. 1999). When animals are subjected to a stressor, the hypothalamus releases the hormone andrenocorticotropic (ACTH), which signals the adrenal cortex to release steroid hormones such as glucocorticoids (GC) to help the individuals to cope with the stressful situation (Sapolsky et al. 2000). Thus, GC concentrations are used as a hormonal measure of physiological stress responses (Wingfield & Romero 2001; Möstl & Palme 2002). The analysis of GC metabolites in faeces has been reported in several vertebrate species as a useful non-invasive technique for assessing the adrenocortical function (Möstl & Palme 2002; Monclús et al. 2006; Lepschy et al. 2007; Barja et al. 2012; Piñeiro et al. 2012; Zwijacz-Kozica et al. 2013; Navarro-Castilla et al. 2014ab).

In mammals, previous studies showed how GC play an important role in responding to diverse factors such as social conflicts, human disturbances and predators (Saplosky et al. 2000; Romero 2002; Barja et al. 2007; Navarro-Castilla et al. 2014ab). Since stressful situations usually evoke an increased GC production, predators could induce physiological responses in their prey by a physical attack but also by making them fearful of an imminent attack (Boonstra et al. 1998; Eilam et al. 1999; Hirschenhauser et al. 2000; Korte 2001; Monclús et al. 2005).

OBJECTIVES

In the present study we tested whether wood mice *Apodemus sylvaticus* showed behavioral and physiological changes due to increased predation risk due to moonlight and exposure to predator odour. Thus, we studied under new moon and simulated full moon conditions and under threat of common genet *Genetta genetta* presence whether these cues for increased predation risk affected foraging activity, food intake and/or physiological stress response in wood mice. Common genet predation is a significant threat for small mammals, especially for wood mice (Hamdine et al. 1993; Virgós et al. 1999), and predator faecal odour has been used previously to simulate predation risk (Dickman & Doncaster 1984; Navarro-Castilla & Barja 2014a). Since variation in predation risk affects foraging decisions (Lima & Bednekoff 1999), we predicted wood mice changing foraging activity and avoiding common genet faeces, especially under high illumination (full moon). We also expected changes in feeding behaviour and

physiological stress responses due to the increased predation risk. We predicted a decrease in food intake under full moon and exposure to common genet faeces, as well as we expected the exposure to predator odour and full moon phase to evoke physiological stress responses in wood mice.

MATERIAL AND METHODS

Study area

Field work was carried out in the savanna-like holm oak *Quercus ilex* woodlands of the National Park of Cabañeros (Central Spain, 30S 385450, UTM 4353479). In this system, large oak trees grow scattered (mean tree density is 14 ha^{-1}) on a grassland matrix with almost no shrub cover (<1%; see Pulido et al. 2001; Díaz et al. 2011).

Experimental design: live trapping and simulation of predation risk

We performed a previous trapping to locate those trees occupied by mice out of the 170 trees growing in two nearby study sites. Afterwards, 40 trees occupied by mice were provided with two Sherman traps. We manipulated direct predation risk through predator odour from one of the rodent predators in the study area, the common genet (*Genetta genetta*). Treatments were interspersed with controls (traps were not treated) by pairing nearby occupied trees and assigning the treatment (traps scented with predator odour) to one tree of each pair at random. Predator treatment consisted in fresh faeces of common genet collected from captive animals of the Cañada Real Open Center (Madrid, Spain). To prevent volatile compounds variation in relation to seasonal or individual factors (Andreolini et al. 1987; Jemiolo et al. 1991; Hayes et al. 2006b; Scordato et al. 2007; Martín et al. 2010), all collected faeces were mixed to obtain a homogeneous mixture avoiding possible bias in our results. Predator treatment was made following methods by Navarro-Castilla & Barja (2014a), 100 g of homogenized faecal sample were mixed with 100 ml of distilled water obtaining a mixture similar to real fresh faeces. Predators' presence was simulated by leaving an equal amount (5 g) of faeces at the entrance of treated traps, and to prevent drying out rapidly, what could decrease its effectiveness, faecal odour was renewed every day at dusk. To test the effect of moon light, traps were operated during five consecutive new moon nights (20-24 March 2012); afterwards, we simulated full moon light conditions at the same sites by means of artificial illumination during the following five nights. The illumination

device was composed of three white and three blue led lights grouped behind a diffusion screen to simulate a diffuse light with the spectral composition of moonlight. It was hanged down from the tree canopy at a height of 2 m to simulate a light intensity of 1 lux (full moon) at ground level, as measured by means of a TES-1332A luxometer. The device was powered by the system used to power video-cameras described below. Nest material (raw wool with natural lanolin) was used as bedding inside traps. All traps were baited with 4 g of toasted corn. Check-ups were carried out every 10–12 h, at dawn and dusk, to minimize the time that animals were kept.

Captured individuals were identified to species. Sex and reproductive condition was determined from external characteristics (Gurnell & Flowerdew 1994). Adult males with enlarged testicles descended into the scrotal sac and females showing noticeable nipples and/or the vaginal membrane perforated were classified as reproductively active. In addition, we used a 100 g hand-held scale to measured body weight which was used to estimate relative age following Navarro-Castilla & Barja (2014a) (juveniles: <13 g; subadults: from 13 g to <20 g; adults: ≥20 g). Individuals were marked in non-conspicuous areas with harmless watery paints (red food colouring: Ponceau-4R E124) for individual identification and to control recaptures. Animals were quickly handled (< 1 min) and then released at the same point of capture. Manipulations of animals were done in compliance with the European Communities Council Directive 86/609/EEC for animal experiments and were carried out under the permit of the Cabañeros National Park authorities.

Mice foraging behavior and food intake

Mice foraging behaviour was videotaped during trapping sessions. The video-recording device was composed by a mini video-camera OmniVision CMOS 380 LTV (3.6 mm lens) focused on Sherman traps by means of tripod 60 cm tall located 1 m away. Video-cameras were provided with ELRO dvr32 card-based recorders, and the whole video-recording set (and the led device used to simulate full moon light) was powered by car batteries (70 Ah, acid-lead) attached to solar panels (ono-silicon erial P_20; 20 w). The system was fully autonomous for continuous recording during three days by setting the recording quality at 5 frames s^{-1} and using 16 GB recording cards. However, it was turned on each day at dusk, before opening traps and renewing predator odour, replacing the memory card by an empty one. Foraging activity was recorded as the time

(s) since individuals appeared in the image and the time they went inside the trap closing it. Video recordings also allow measuring the time spent by each individual inside traps. Bait remains were dried at 50 ºC in a laboratory heater (Selecta, model CONTERM 2000208) to remove dampness and weighed with an electronic balance (Giros PG-500; precision 0.01 g) to determine the amount of food eaten. Food intake for each individual was divided by its body mass to avoid any influence of this variable on food intake.

Faeces collection and faecal corticosterone metabolites (FCM) quantification

We collected fresh faeces from traps where individuals were captured, excluding faecal samples if urine was detected in order to avoid cross contamination (Touma et al. 2003). To avoid environmental conditions and microorganisms proliferation effects on FCM levels (Washburn & Millspaugh 2002; Millspaugh et al. 2003), we only collected fresh faeces between sunrise and two hours after. Thus, we also avoided circadian rhythm effects on excretion patterns (Touma et al. 2003). Since corticosterone concentrations were observed in laboratory mice faeces up to 10 h after the ACTH injection (range: 8–12 h; Touma et al. 2004), traps were reviewed within 10 h after activation to avoid a possible effect of the capture in FCM levels. Faecal samples were stored in the freezer at -20º C until analysis.

Extraction of FCM from faecal samples was done according to the modified method of Touma et al. (2003). Faecal samples were unfrozen and dried until constant weight. We placed 0.05 g of dry faeces in assay tubes with 0.5 ml of phosphate buffer and 0.5 ml of 80% methanol, then, they were shaken for 16 h and supernatants were centrifuged at 2500 g for 15 min. Pellets were discarded and the faecal extracts were stored at –20ºC until analyzed. We used a commercial corticosterone enzyme immunoassay (ELISA DEV9922; DEMEDITEC Diagnostics GmbH, D-24145 Kiel, Germany) for the quantification. Parallelism, accuracy and precision tests have to be done to validate any enzyme immunoassay (Goymann et al. 1999; Young et al. 2004). Parallelism was performed with serial dilutions of faecal extracts (1:32, 1:16, 1:8, 1:4, 1:2, 1:1) resulting in a curve parallel to the standard. Accuracy (recovery) was 118.6 ± 31.7 % (n=6). Precision was tested through intra- and inter-assay coefficients of variation for three biological samples, being 4.7 % (n = 6) and 8.2 % (n = 3), respectively. In each assay, we used a standard, whose corticosterone metabolite

concentration was known, included in the DEMEDITEC kit. The assay was excluded and samples were reanalysed if standard corticosterone metabolite concentrations deviated more than 10% from the expected value. The assay detection limit (sensitivity) for corticosterone metabolites was 4.1 ng/mL. FCM levels are expressed as ng/g dry faeces.

Data analysis

Capture frequencies according to odour and moonlight treatments, as well as their interactions with individual characteristics (sex, age and breeding condition) were analyzed by fitting log-linear models to the five-way contingency table generated by the factors odour (control/ common genet faeces), moonlight (new moon/simulated full moon), sex/age (adult male, adult female or juvenile), breeding condition (active or not) and presence/absence of capture, taking into account the structural zeros resulting from the impossibility of finding sexually active juveniles (Díaz et al. 1999; Morán-López et al. 2015). Recaptures were not taken into account in the captures frequencies tests to maintain data independence.

We used General Linear Models (GLMs) to analyse differences in foraging behaviour (s) due to moon phase, treatment (control / faecal odour), sex, breeding condition (breeding / non-breeding), age (juveniles / sub-adults / adults) , recapture (new capture / first recapture / second recapture / third recapture). Equally, we performed GLMs to test variation in food intake (corrected by animal´s body weight), fixed factors were the same as in the foraging activity model (except age factor) and we included the time that each individual spent inside the trap as a covariable. Finally, variation in FCM was analyzed by GLMs, including moon phase, treatment, sex, breeding condition and recapture as fixed factors and body weight of individuals was included as a covariable. Variables were log-transformed as needed to normalize distributions. Results are given as mean ± standard error (SE). We used the SPSS 15.0 statistical software (SPSS Inc, Chicago, IL, U.S.A.).

RESULTS

Overall, 153 wood mice were live-trapped (71 new captures; 82 recaptures). The study population was dominated by adults (80.3% vs. 19.7%) and it was female-biased (56.1% vs. 43.9; Table 5). In addition, reproductive activity was also female-biased

(53% active females vs. 20% active males; Table 5). Regarding predation risk factors, the simulated full moon decreased the number of captures compared to the new moon phase (36.5% vs. 63.5%, respectively) and the treatment with genet faeces also decreased overall captures (46.5% vs. 53.5% in control traps), but pure effects of the odour treatment were not significant (Table 5, Fig. 7A). Nevertheless, we found a significant interaction between treatment*sex/age*breeding condition (Table 1) showing that non-breeding adult males clearly avoided common genet faeces (χ^2 = 7.04, df = 1, P = 0.008; Fig. 7B). No interactive effects among predator risk factors were significant (Table 5).

Table 5. Results of the fit of log-linear model analyzing the effects of individual and predation risk factors on the capturability of wood mice

Effect	**df**	**G^2**	***P***
Sex/age	2	7.38	0.025
Breeding condition	1	10.68	0.001
Sex/age*Breeding condition	1	6.66	0.010
Treatment	1	0.36	0.548
Moonlight	1	12.35	0.000
Moonlight*Sex/age	2	3.03	0.220
Moonlight*Breeding condition	1	0.15	0.695
Treatment*Moonlight	1	0.04	0.843
Treatment*Sex/age	2	1.94	0.379
Treatment*Breeding condition	1	0.21	0.644
Treatment*Moonlight*Sex/age	2	0.42	0.809
Treatment*Moonlight*Breeding condition	1	0.14	0.712
Treatment*Sex/age*Breeding condition	1	6.54	0.011
Moonlight*Sex/age*Breeding condition	1	1.01	0.316
Treatment*Moonlight*Sex/age*Breeding condition	1	0.97	1.000

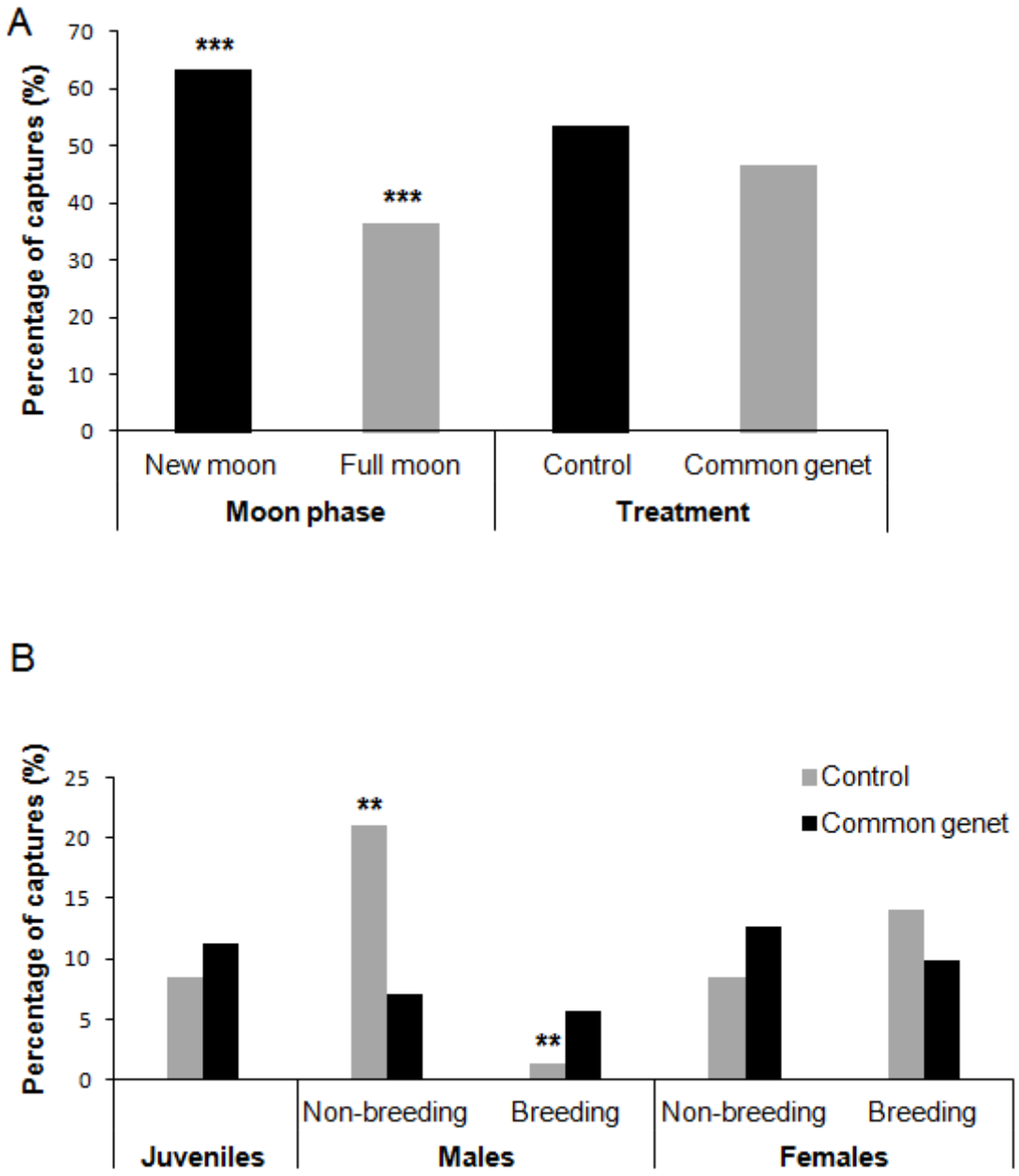


Figure 7. Percentage of wood mice captured in relation to direct (common genet faeces) and indirect (moon light) cues of predation risk (A). Percentage of captures according to treatment, sex/age and breeding condition (B). Asterisks indicate significant differences between the analyzed groups (** $p < 0.01$; *** $p < 0.001$).

Mice foraging behaviour and food intake

Treatment was the only significant factor explaining the variation found in foraging activity before entering traps (Table 6). Individuals spent less time to enter treated traps (24.56 ± 2.60 s) than when they faced the control ones (31.54 ± 4.67 s; Table 6, Fig. 8). In relation to the amount of food consumed, only breeding condition resulted as a

significant factor (Table 7). Breeding individuals showed a lower food intake (0.091 ± 0.008 g/g) than the non breeding ones (0.174 ± 0.013 g/g) (Table 7). In addition, breeding individuals decreased food intake when facing common genet treated traps whereas non-breeding individuals increased food intake as compared to control traps (Fig. 9). However, neither this interaction nor other factor interactions resulted statistically significant. Regarding moonlight´s influence, we found an unexpected increase in food intake during artificial full moon conditions, but this effect was no significant only (Table 7). Contrary to what we previously expected, the time that animals spent inside traps did not influence food intake (Table 7). Finally, although recapture effects were not significant, we found that recaptured individuals dedicated less time to forage and they also showed a higher food intake compared to those individuals captured for the first time.

Table 6. Results of the GLMs testing for the effects of predation risk and individual factors on wood mice foraging behaviour.

Factor	**F**	**df**	***P***
Moon phase	0.301	1	0.587
Treatment	6.945	1	0.013
Sex	1.176	1	0.286
Breeding condition	2.554	1	0.120
Relative age	1.680	1	0.202
Recapture	1.366	1	0.271

Table 7. Food intake by wood mice in relation to predation risk and individual factors.

Factor	**F**	**df**	***P***
Moon phase	3.579	1	0.065
Treatment	1.432	1	0.238
Sex	0.019	1	0.890
Breeding condition	8.486	1	0.006
Recapture	6.563	1	0.231
Time inside trap	2.608	4	0.114

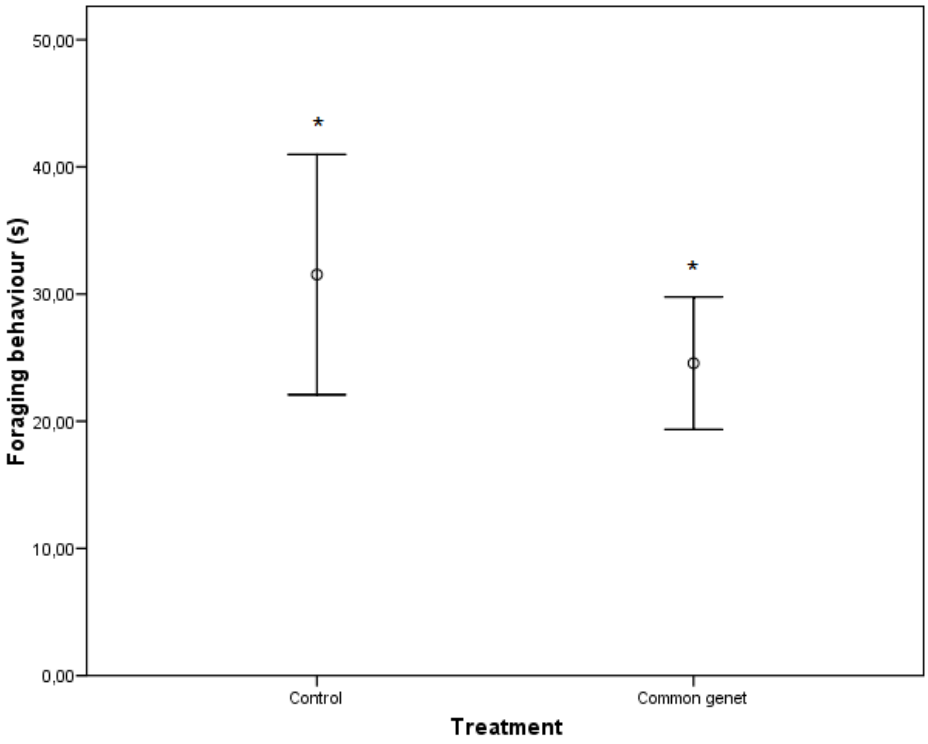


Figure 8. Effect of treatment (control / common genet) on wood mice foraging behavior. Significant differences are indicated by asterisks (*p<0.05).

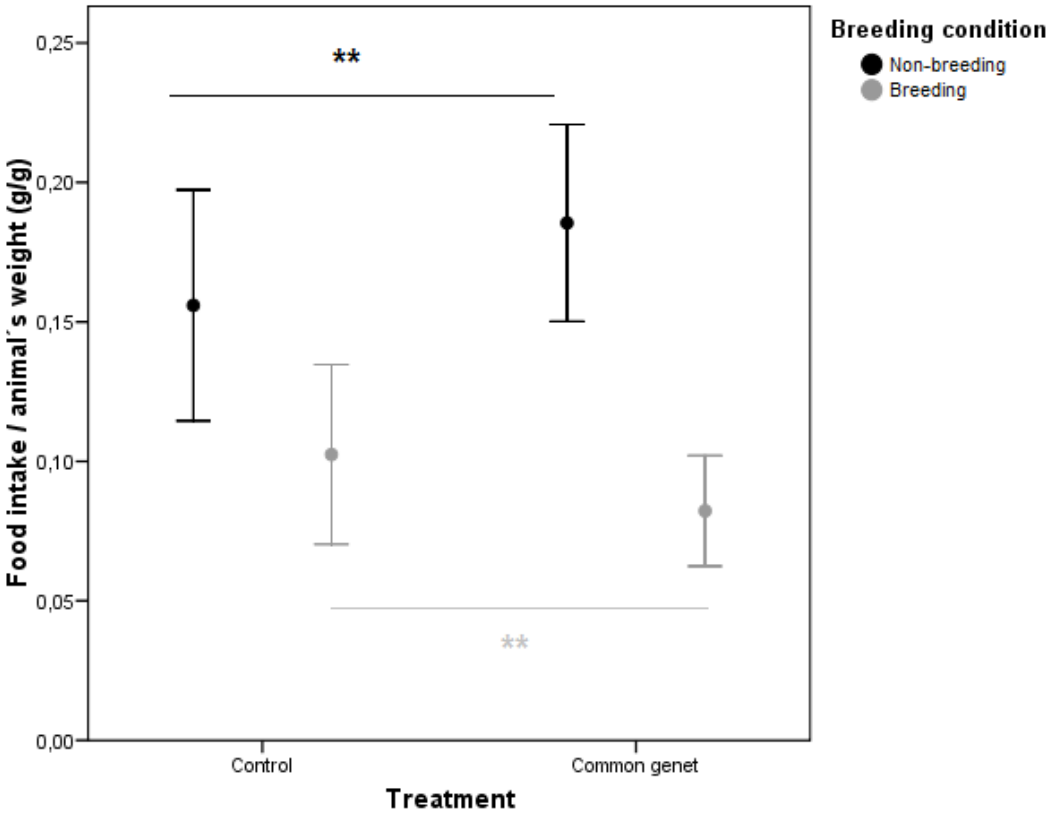


Figure 9. Food intake differences due to breeding condition of individuals. Asterisks represent significant differences between the analyzed groups (**p<0.01).

Faecal corticosterone metabolites (FCM)

FCM levels were analyzed in 107 fresh faecal samples. Factors explaining the variation found in FCM concentrations are presented in Table 8. Body weight of individuals was positively correlated with FCM levels (Table 8). We found higher FCM concentrations in breeding individuals (262285 ± 97433 ng/g dry faeces) compared to the non breeding ones (85502 ± 24165 ng/g dry faeces). However, differences in FCM levels due to breeding condition were not statistically significant (Table 8). FCM levels were lower in males (130695 ± 53407 ng/g dry faeces) than in females (138762 ± 41306 ng/g dry faeces) and individuals showed lower FCM levels when they were recaptured (84928 ± 35891 ng/g dry faeces) than when they were captured for first time (165002 ± 47982 ng/g dry faeces). However, the interaction between sex*recapture disclosed that significant differences in FCM were between females captured for the first time and recaptured males (Table 8, Fig. 10). Neither moon phase nor predator odour resulted as significant factors influencing FCM levels.

Table 8. Results of the General Lineal Model testing the effects of individual and predation risk factors on faecal corticosterone metabolites in wood mice.

Factor	**F**	**df**	***P***
Moon phase	2.690	1	0.105
Treatment	0.025	1	0.875
Sex	0.177	1	0.675
Breeding condition	0.170	1	0.681
Recapture	1.235	1	0.270
Body weight	5.660	1	0.020
Sex*Recapture	4.178	1	0.044

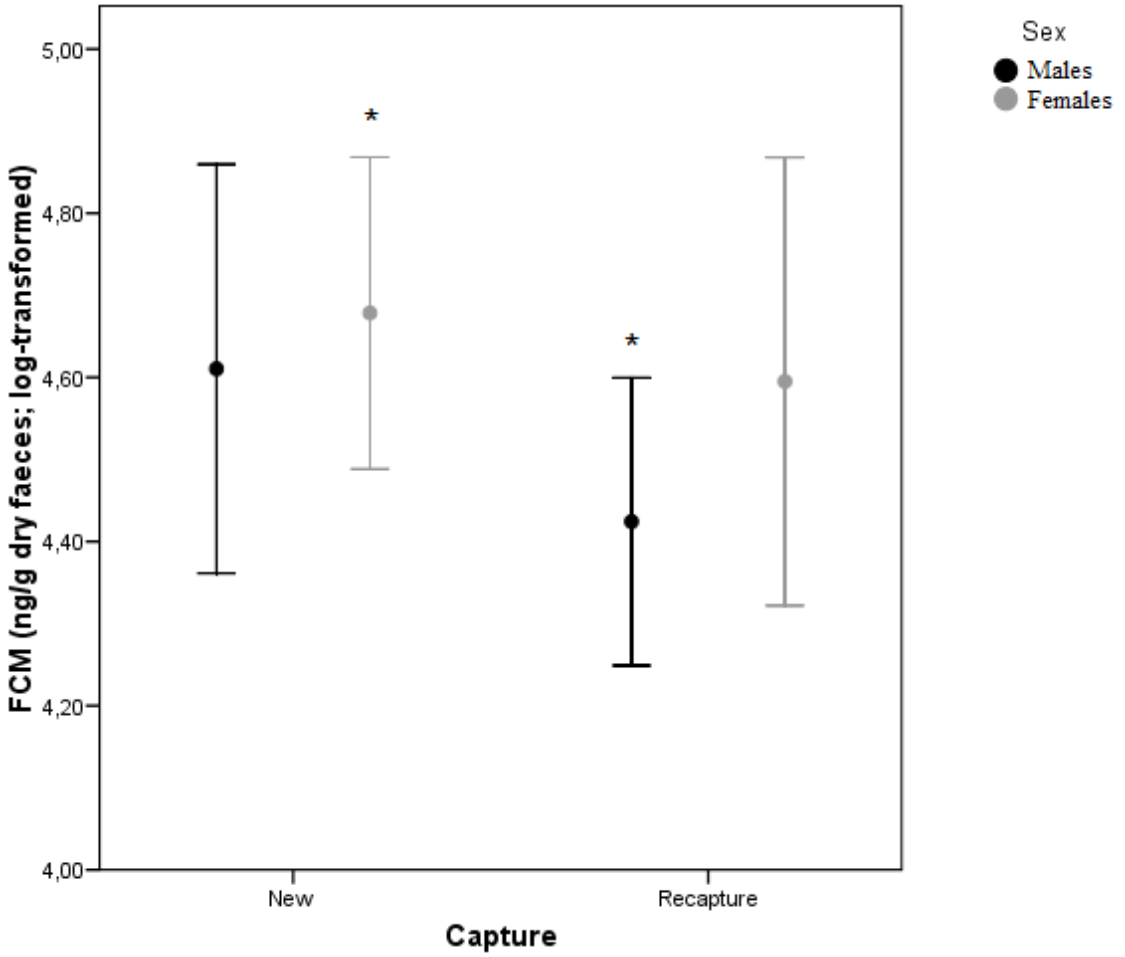


Figure 10. Log-transformed concentrations (mean ± SE) of faecal corticosterone metabolites (FCM, ng/g dry faeces) in males and females in relation to new captures or recaptures.

DISCUSSION

Wood mice were less captured during the simulated full moon due to a decrease in activity as was previously described in this and other prey species (Kotler et al. 2010; Penteriani et al. 2013; Navarro-Castilla & Barja 2014b). Therefore, moonlight can indicate a higher risk of predation since individual´s vulnerability to a predator depends partly upon visibility. On the other hand, predator odour might provide direct information on predation risk even when the predator is absent at the time of detection. In this study, the number of captures decreased in traps treated with common genet faeces, coincident with several studies where avoiding areas marked by predators was common in small mammals (Dickman & Doncaster 1984; Calder & Gorman 1991; Russell & Banks 2007). However, this antipredatory response varied depending on individual characteristics, being significantly different from random expectations for

non-breeding adult males only. Coincident with these results, Dickman & Doncaster (1984) showed that male wood mice exhibited a higher avoidance of predator faeces than females did. However, breeding males did not show such avoidance being even more captured in predator treated traps. Their territorial behaviour during the breeding season (Montgomery & Gurnell 1985) and their attraction for new objects (Brown 1969) could be a possible explanation. Similar differences in response to predator cues due to sex and breeding condition were also found in bank voles (*Clethrionomys glareolus*) by Jedrzejewski & Jedrzejewska (1990). Therefore, sex and breeding condition differences in the responses to predation threats suggest that gonadal hormones may be involved in the mediation of the antipredatory responses (Perrot-Sinal et al. 1999). On the other hand, young mammals typically devote less time to predator detection (Arenz & Leger 2000) what could explain why juveniles were equally captured although predation risk increased. Therefore, while indirect risk cues (moonlight) seems to be perceived by most population as more reliable proofs to value predation risk situations (Orrock et al. 2004), responses to direct cues (predator faeces) are not a generalized behaviour depending on individual characteristics and probably explained by previous experience (Lima & Bednekoff 1999).

Mice foraging behaviour and food intake

Predation may influence animal daily decision making to choose when, where and how long to forage. According to Lima and Bednekoff (1999), under high risk situations prey reduce time spent in daily activities to optimize the energy spent on antipredatory behaviour. Thus, several studies have shown how individuals under high levels of risk decreased mobility concentrating foraging activity in safer habitats (Lima & Dill 1990; Díaz 1992; Kotler et al. 2002). Our results also showed predation risk influencing foraging behavior. We found that individuals facing traps treated with common genet faeces reduced foraging activity outside traps by going inside them faster than those individuals captured in the control ones. However, no effect of moonlight was found on foraging activity. These results perfectly match with Díaz et al. (2005), who found that wood mice reduced foraging behaviour in response to the presence or activity of common genets but there was no effect of moon light on foraging activity. According to Lima & Bednekoff (1999), animals under longer periods of high risk (e.g. full moon nights) are forced to decrease antipredatory behaviour and forage to meet their energy

demands. This hypothesis could explain why although full moon is supposed to increase perceived predation risk and wood mice were expected to decreased food intake, however, they increased food intake during full moon nights. Alternatively, as full moon samplings were carried out after new moon, the increase in food intake during full moon might potentially be an effect of recapture, which is discussed below. Anyway, preys have to trade off food and safety under each situation, but they have also to prioritize between different daily activities. Thus, breeding individuals could be more careful under risky situations reducing feeding and allocating more time to survive and breed. Finally, we found that being recaptured decreased foraging time and led to a higher food intake. These results could be explained by the previous experience. Thus, animals facing up traps for the first time should be more stealth, however, after that successful first time individuals would positively value the benefits of obtaining food than the risk perceived.

Physiological stress responses

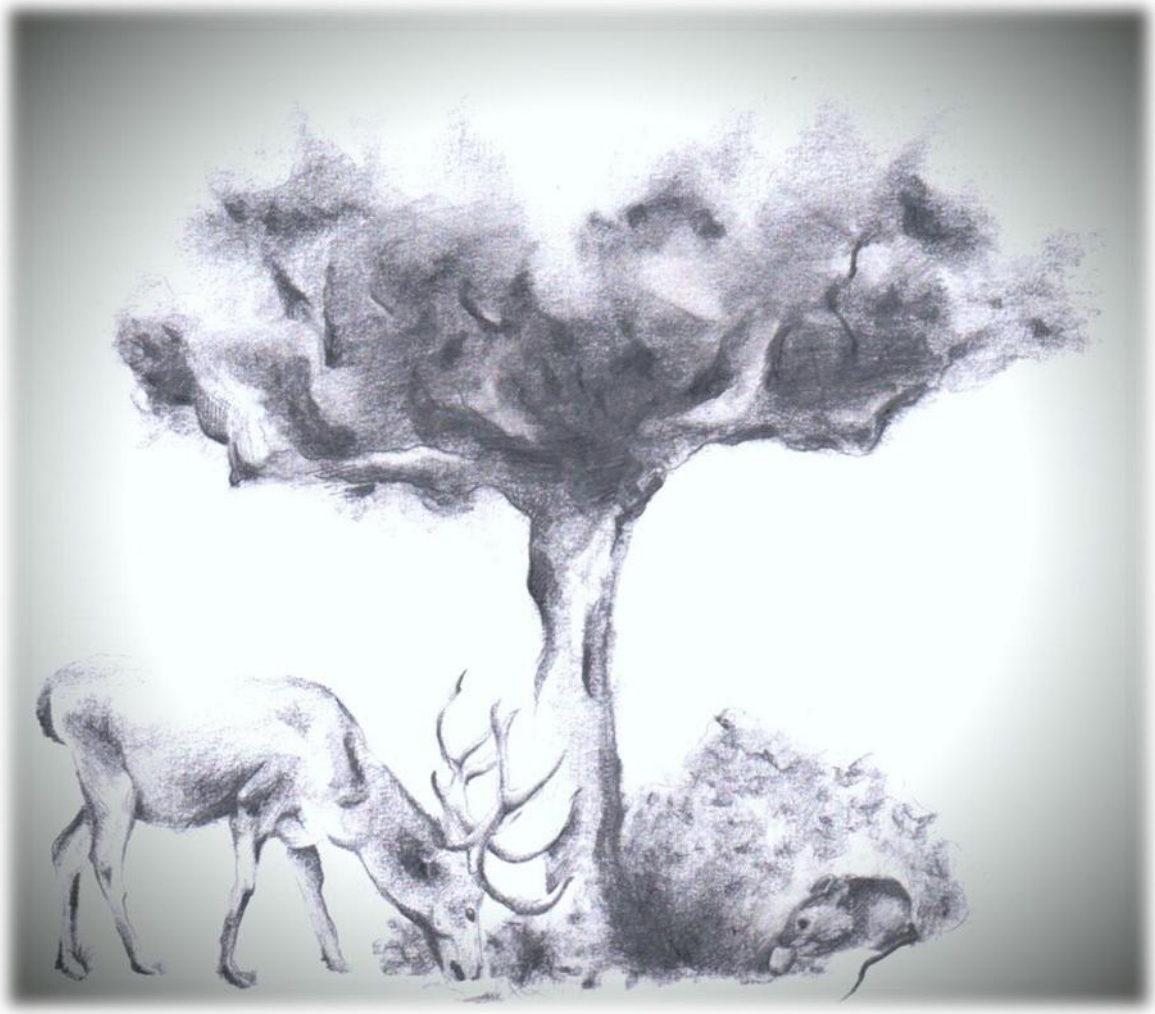
Although differences were not statistically significant, the higher FCM levels found in breeding individuals are coincident with studies done in different mammal species (Romero 2002; Dantzer et al. 2010; Navarro-Castilla et al. 2014ab). Increased glucocorticoid concentrations during breeding have been attributed to metabolic changes due to pregnancy and lactancy (Bauman 2000; Strier et al. 2003; Reeder & Kramer 2005) as well as to the increased competition and aggressive behaviour in males. Females showed higher FCM levels than males. This is in accordance with previous studies in this and other rodent species (Touma et al. 2004; Navarro-Castilla et al. 2014ab) and it could be mainly attributed to differences in the metabolism and/or excretion of glucocorticoids between both sexes (Touma et al. 2003). However, since the interaction between sex*recapture showed a significant influence on FCM levels, this could also indicate a greater stress response by females to the novel testing environment. In addition, we found that body weight of individuals, which is closely related with the age of individuals (Gurnell & Flowerdew 1994), was positively correlated with FCM levels. Adults exhibited higher FCM levels maybe as a consequence of the above mentioned breeding condition and increased aggressiveness. Alternatively, individuals might simply display age-related physiological responses to cope with stressors (Hauger et al. 1994).

Physiological responses due to simulated predation risk by owl calls were previously found for voles and mice (Eilam et al. 1999). However, studies where predation risk was simulated with predator odour did not evoke any physiological response in different rodent species (bank voles and weasels: Ylönen et al. 2006; meadow voles and weasels: Fletcher & Boonstra 2006). In the present study, neither moonlight nor exposure to predator odour had any influence on FCM in wood mice, a result similar to the lack of effect of moonlight and red fox faecal odour on the physiological stress response of wood mice found by Navarro-Castilla & Barja (2014b). Direct and indirect cues of predation risk do not seem to be enough to elicit physiological responses leading to increased FCM levels or, alternatively, this lack of response would prevent individuals from suffering the negative physiological consequences of chronic stress on fitness (Möstl & Palme 2002).

In summary, wood mice behavioural changes under perceived predation risk situations probably reduce the probability of an encounter with a predator, but they imply important trade-offs between the benefits of safety from predation and the costs associated to missing opportunities for foraging or reproduction (Abrams 1986; Lima & Dill 1990; Brown et al. 1999; Brown & Kotler 2004). Common genets defecate in latrines their faeces may be less indicative of their presence or movement patterns, and therefore, a generalized antipredatory response would not lead to large survival benefits relative to the costs of losing food. Thus, wood mice are expected to exhibit different antipredatory responses only when they have an accurate assessment of the current predation risk and making decisions choosing those behavioral options which maximize their fitness. Therefore, predation risk is taken into account in the decision making process by wood mice and individuals have evolved different antipredatory responses to threatening stimuli. Nevertheless, they seem to respond when those stimuli cross a particular threshold and behavioral responses depend on context, past experience and the status of each individual (Gorman & Trowbridge 1989). Regarding the lack of physiological response to predation risk by mice, moonlight and predator odour could not be reliable enough to outweigh the associated energetic costs of such responses. So, making decisions by altering behavioural responses seems to be better, faster and more useful options to maximize fitness.

CHAPTER 2

INTERSPECIFIC COMPETITION



PAPER IV

Navarro-Castilla, Á., Díaz M. & Barja, I. (2015). Does ungulate disturbance mediate behavioral and physiological stress responses in Algerian mice (*Mus spretus*)? A wild exclosure experiment. *Zoology* (under 2nd review).

PAPER IV

DOES UNGULATE DISTURBANCE MEDIATE BEHAVIORAL AND PHYSIOLOGICAL STRESS RESPONSES IN ALGERIAN MICE (*Mus spretus*)? A WILD EXCLOSURE EXPERIMENT

ABSTRACT

Ungulate densities increased recently in Mediterranean oak ecosystem where acorn-dispersing small rodents are keystone species. Recent work showed effects of ungulates on small mammal behaviour and population dynamics, but little is known on the physiological stress responses of small mammals to ungulates. We manipulated ungulates´ presence by large exclosures in Holm oak *Quercus ilex* open woodland with paired controls. We combined live trapping with a detailed evaluation of habitat features such as vegetation height, cover and soil compaction. To test wether ungulate presence led to increased glucocorticoids, we analysed faecal corticosterone metabolites (FCM) in wild populations of Algerian mice (*Mus spretus*). Fresh faecal samples from 92 different individuals captured with Sherman live traps were collected and analyzed by an enzyme immunoassay. Mice abundance was higher inside ungulate exclosures due to positive exclosure effects on understory vegetation cover and soil compaction. Mice selected smaller trees with more area covered by resprouts, and this selection was stronger outside than inside exclosures. FCM levels were higher in females than in males. FCM levels were positively correlated with soil compaction and inversely with tree canopy size, however, individuals showed higher FCM levels inside than outside exclosures due to the higher local mice abundance inside exclosures. Mice behaviour varied in relation to direct effects of wild ungulates on key habitat traits whereas physiological stress responses seemed to be mediated by the increased intraspecific competition, an indirect effect of wild ungulates.

Keywords: faecal corticosterone metabolites, physiological reactions, rodents, soil compaction, vegetation cover, ungulate impacts

INTRODUCTION

Grazing by large herbivores, either wild or domestic, may greatly alter nearly every aspect of the local environment. The ecological effects of grazing include the alteration of the composition of communities, the disruption of ecosystem functions, and the alteration of ecosystem structure (Franklin et al. 1981). Previous studies have shown that ungulates can significantly impact the ecosystem by affecting the floristic species composition, richness, vertical profiles, plant traits, soil erosion and compaction, water infiltration and a number of other attributes (Milchunas et al. 1988; Noy-Meir et al. 1989; McIntyre & Lavorel 2001; Rodríguez et al. 2003; Torre et al. 2007). Coincident with these vegetation changes caused by ungulates are changes in animal communities. As over-grazing implies important changes in the habitat, ungulates also have the potential to influence the diversity of consumers ranging from insects to birds and mammals (Grant et al. 1982; Bock et al. 1984; van Wieren 1998; Fuller 2001; Stewart 2001; Torre et al. 2007). The effects of ungulates on vegetation and soil will affect mainly those animal species that depend on vegetation for food and rely on plant cover and soil for foraging safely and/or for refuge (Milchunas et al. 1988; Keesing 1998; Eccard et al. 2000). For instance, small mammals are strongly influenced by habitat structure (Rosenzweig & Winakur 1969; Bowers & Flanagan 1988) and habitat selection is mainly based on specific components such as favorable microclimates, food resources, avoidance of competitors and cover as protection from predators (Brown et al. 1988; Brown 1989). Because even moderate grazing by large herbivores reduces vegetation cover drastically, there can be little doubt that small mammal communities could be affected by ungulates (Torre et al. 2007); Muñoz et al., 2009). Increasing densities of ungulates and the associated increase in grazing pressure may have far-reaching impacts in small mammal ecology. These possible impacts occur through two different ways. Habitat modification or removal due to reductions in cover is assumed to increase the exposure to predators (Birney et al. 1976; Smit et al. 2001; Schmidt & Olsen 2003; Orrock et al. 2004), and grazing may also influence small mammals directly by physical disturbance such as trampling of soil and browsing (Grant et al. 1982; Bock et al. 1984; Hayward et al. 1997; Torre et al. 2007) and by rooting in the case of wild boar (Focardi et al. 2000); these factors affect food supply, cover and the balance of competition between species. Ungulates may also compete for food with

small mammals (Keesing 1998; Focardi et al. 2000; Caro 2002; Schmidt et al. 2005) and even predate occasionally on them in the case of wild boars (Herrero et al. 2006). Therefore, clear-cut responses of small mammals to grazing are to be expected.

Most studies on the responses of small mammals to disturbance by ungulates have focused on changes in population (e.g. density) or community (e.g. richness, abundance) parameters in comparisons of captures between grazed and ungrazed areas, showing mixed effects on small mammals according to the species (Grant et al. 1982; Bock et al. 1984; Putman et al. 1989; Heske & Campbell 1991; Hayward et al. 1997; Jones & Longland 1999; Jones 2000; Moser & Witmer 2000; Giuliano & Homyack 2004; Valone & Sauter 2005). However, we have not found available papers on the likely physiological basis of these population or community level responses. Ungulate activity may in fact act as a potential environmental stressor to individual mice, causing physiological stress reactions that could influence population dynamics and behavioral changes, potentially influencing plant population's dynamics.

In mammals, diverse factors such as predators, social conflicts, human disturbances, interspecific competition, and even capture, cause stress reactions (Boonstra et al. 1998; Creel et al. 2002; Sands & Creel 2004; Barja et al. 2007; Piñeiro et al. 2012; Zwijacz-Kozica et al. 2013). When an animal is subjected to a stressor, glucocorticoid concentrations tend to increase thus indicating a physiological stress reaction (Wingfield & Romero 2001). Glucocorticoids can be quantified in plasma, urine, saliva and faeces (Sheriff et al. 2011). Faecal glucocorticoid quantification is specially useful as it is a non-invasive method that avoids the additional stress that can be caused by animal capture, handling, sedation and/or transport (Sheriff et al. 2011). In fact, this method has been reported as a useful technique in wild vertebrates such as rodent species (Navarro-Castilla et al. 2014ab), red squirrels *Sciurus vulgaris* (Dantzer et al. 2010), European pine martens *Martes martes* (Barja et al. 2007), wolves *Canis lupus* (Barja et al. 2008), European badgers *Meles meles* (Barja et al. 2012) and wildcats *Felis sylvestris* (Piñeiro et al. 2012). Physiological stress responses to environmental disturbance are adaptive in the short term; however, if glucocorticoid levels remain elevated for longer periods of time ('chronic stress'), a wide range of negative consequences can occur, including immunosuppression, reproductive suppression, and tissue atrophy (Sapolsky 1992; Sapolsky et al. 2000; Sapolsky 2002; Stewart 2003).

These negative consequences increase animals' vulnerability, compromising their survival and reproductive capacity (Lochmiller & Deerenberg 2000) and therefore, affecting their fitness (Möstl & Palme 2002).

Ungulate densities, either wild or domestic, have increased recently in managed Mediterranean oak ecosystems, the dehesas (Díaz et al. 1997; Perea et al. 2014), where they seem key to explain the general regeneration failure of this high-value system. Negative ungulate effects on regeneration have been proposed to be both direct, by consumption and trampling of acorns and seedlings (Gómez et al. 2003; Pulido & Díaz 2005; Perea & Gil 2014b), and indirect, by influencing the effectiveness of acorn dispersal to safe sites for seedling (Smit et al. 2008; Smit et al. 2009). In fact, the relationship between oaks and mice is a conditional mutualism, whose outcome can change to antagonism (seed predation) when external factors such as intra-and interspecific competition influence hoarding behavior and cache retrieval (Morán-López et al. 2015). This latter will be a case of cascading effects of ungulates on oak population dynamics (Schmitz 2008).

OBJECTIVES

In this study we investigated the impact of wild ungulates on rodent populations in Mediterranean man-made dehesas. Given habitat and diet overlap, and given that the shared resources are in limited supply (Tokeshi 1999), if competition and interaction between ungulates and rodent species are important, we hypothesized that ungrazed ungulate exclosures would provide a better quality habitat with more resources (food and cover) and less compacted soils than available in grazed plots. Thus, we predict that rodent abundances should increase in the absence of ungulates. We also studied how ungulates affected soil compaction, tree size and cover and height of understory vegetation and whether habitat selection by mice was affected by these habitat traits. Based on these attributes, mice will be expected to select less compacted areas. In addition, a higher cover and height of understory plants is expected to determine tree occupancy by mice. On the other hand, small mammal physiological stress responses to ungulate presence have not been previously described. We collected small mammal faeces and quantify faecal corticosterone metabolites (FCM) as indicators to test whether the presence of ungulates has any physiological influence on small mammal populations. If ungrazed areas are better foraging habitats for small mammal

populations, we predict that animals inhabiting and using these less disturbed ungrazed enclosures would have lower levels of FCM.

MATERIAL AND METHODS

Study area

Field work was conducted between November 2010 and March 2012 in the Cabañeros National Park (Ciudad Real province, Central Spain, 30S 385450, UTM 4353479), established in 1995 as a natural reserve representative of the Mediterranean vegetation of the Iberian Peninsula. Climate is characterized by summer drought (Díaz et al. 2011) with a mean annual rainfall of 636 mm and mean annual temperature is 15.0 ºC. Vegetation is dominated by Mediterranean oak forests (*Quercus* spp.) and shrublands. Forests located in the plain lowlands of the park were opened in the 1960s to create dehesas, which are man-made savannah-like woodlands with scattered trees (mean tree density is 14 trees ha^{-1}) growing in an open grassland matrix with almost no shrub cover (<1%; see Pulido et al. 2001; Díaz & Pulido 2009; Díaz et al. 2011).

Two species of rodents, the Algerian mouse *Mus spretus* and the wood mouse *Apodemus sylvaticus*, inhabit the study area (Muñoz et al. 2009). Both species are prominent predators and dispersers of acorns during the acorn fall season (Pulido & Díaz 2005; Muñoz & Bonal 2007). Individual mice select positively oak canopies in dehesas as both refuge areas and foraging grounds, specially during fall-winter, when its staple food are the acorns (Muñoz et al. 2009). Red deers *Cervus elaphus* occupy dehesas and forests at densities of 0.13-0.14 individuals/ha (Jimenez 2004). Wild boars *Sus scrofa* are also common (Perea & Gil 2014a), whereas roe deers *Capreolus capreolus* are restricted to humid pine and oak forests (Díaz et al. 2004). Mammalian carnivores such as common genets *Genetta genetta*, stone martens *Martes foina*, red foxes *Vulpes vulpes*, badgers *Meles meles* and wild cats *Felis sylvestris* are common, as ascertained by scat searches, as were barn owls *Tyto alba*, kestrels *Falco tinnunculus* and harriers *Circus cyaneus*.

Ungulate exclosures

Mice trapping and vegetation and soil measurements were performed in two large ungulate exclosures paired with nearby grazed areas with similar tree density, distribution and cover (see Díaz et al. 2011 for details). Sites were separated 1500 m.

One exclosure was established in 1995 covering an area of ca 150 ha, from which we selected its southernmost 3 ha section including 50 oak trees and an equivalent contiguous grazed area with the same size and shape, also including 50 oak trees. The other exclosure was built in 2008 with 36 oak trees inside 4.65 ha, paired with a grazed area of 4.65 ha with 34 oak trees (see Díaz et al., 2011 for a detailed map). Exclosures were built with wire fences 2 m tall and 32 cm x 16 cm mesh width. Both exclosures allowed free passage by mammalian rodent predators, as ascertained by scat searches inside and outside (Muñoz & Bonal 2007); avian predators are not excluded by fences.

Animal trapping and data collection in the field

We sampled rodent communities within ungulate exclosures and their paired grazed plots. Four trapping sessions were carried out in November 2010 and 2011 and in February 2011 and 2012, and live trapping was done during new moon to maximize rodent trappability (Díaz 1992). Traps were located under all trees present in the study sites, as mice barely used the open grassland matrix (Muñoz et al. 2009). A total of 170 oak trees, located inside and outside exclosure plots (details above), were provided with two standard Sherman live traps that were operated during two consecutive nights, with a total effort of 2720 traps-night. Traps were baited with a mixture of tuna and flour and with a piece of apple. Traps were also partly filled with waterproof cotton wool to improve thermal insulation. They were set at dusk and examined the following morning.

Captured individuals were identified to species and weighed (to the nearest 1 g) using a spring balance. Sex and reproductive condition was determined from external characteristics (Gurnell & Flowerdew 1994). In breeding adult females the nipples on the abdomen and thorax are noticeable and the vaginal membrane appears perforated, whereas breeding adult males present the testicles enlarged quite markedly and usually descended into the scrotal sac. All captured individuals were marked with numbered ear tags to identify and exclude recaptures from data analyses in order to achieve sample independence. Individuals were handled as fast as possible and they were released at the same point of capture. In this research, we fulfilled all the regulations concerning to handling and treatment of animals in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for animal experiments and manipulations of animals were done under the permit of the authorities of the National park.

Vegetation variables and soil compaction

The size of the canopy projection of each tree and the area of resprouts under it was estimated by measuring their diameters in N-S and E-W directions. We estimated sizes assuming a roughly circular shape of both the canopy and the resprout patch, as resprouts always encircle tree trunks. Mean height of the resprout patches was measured to the nearest cm with a ruler put vertically on the ground. Local tree density (and its inverse, tree isolation) was measured as the cover of oak canopies in 50-m circles centered on focal trees minus the canopy of such focal trees. Densities were estimated by means of SIG analyses on georeferenced high-resolution (1:5000) aerial photographs. Soil compaction was measured in November and February on six regular sampling grids of 10 measurement sites each within each exclosure and in the corresponding adjacent grazed plots. Measurement sites within sampling grids were arranged in two rows of 5 measurement sites spaced 10 m, and grids within exclosures and grazed plots were set every 70 m, thus ensuring that measurement sites were interspersed under tree canopies and in the open grassland to account for microhabitat effects (see Díaz et al., 2011 for details). In each site we measured soil compaction at five points, one in the site and the other four 1 m away in the four cardinal directions. We measured soil compaction at these five points as the force (in N) needed to introduce a steel rod (diameter: 6 mm) with a conical point (length: 6 mm) 20 mm into the soil (Torre et al. 2007). The force was measured with a Mecmesin® BFG 500N electronic force gauge to the nearest 0.1 N. Soil compaction values for the trees occupied by mice were estimated as the mean values of the 25 compaction measures taken in the five nearest measurement sites.

Faeces collection and quantification of Faecal Corticosterone Metabolites (FCM)

Fresh faeces were collected only between sunrise and two hours thereafter to assure that exposure to environmental conditions and microbial action was minimized (Millspaugh & Washburn 2003; Möstl et al. 2005; Barja et al. 2012) and to avoid the influence of circadian rhythm in excretion patterns (Touma et al. 2003; Touma et al. 2004). Faecal samples from traps where urine was detected were excluded to prevent a possible cross contamination. Faecal samples were stored in the freezer at -20º C until analysis.

FCM were extracted from faecal samples according to the modified method of (Touma et al. 2003). Frozen faecal samples were dried at 50ºC until constant weight,

and then 0.05 g of dry samples were placed in assay tubes with 0.5 ml of phosphate buffer and 0.5 ml of pure methanol. Tubes were shaken for 16 h and the supernatants were centrifuged at 2500 r.p.m. for 15 min. Pellets were discarded and the faecal extracts were stored at –20ºC until analysis. A commercial corticosterone enzyme immunoassay (ELISA DEV9922; DEMEDITEC Diagnostics GmbH, D-24145 Kiel, Germany) was used for the quantification. The cross-reactivity of the antibodies with other substances according to the manufacturer was lesser than 2.4%.

Three tests have to be done to validate any EIA: parallelism, accuracy and precision (Goymann et al. 1999; Young et al. 2004). A parallelism test of serial dilutions of extracts was performed with dilution ratios of 1:32, 1:16, 1:8, 1:4, 1:2, 1:1, and a curve parallel to those of the standard ($p > 0.05$) was obtained. Recovery (accuracy) was 100.1 ± 34.8% (n = 4). Intra- and inter-assay coefficients of variation (precision) were calculated with extracts and the obtained values were 4.7 % (n = 6) and 8.2 % (n = 3), respectively. In each assay, we used a standard (50 ng/mL corticosterone) included in the DEMEDITEC kit. When standard corticosterone metabolite concentrations deviated more than 10% from the expected value, the assay was rejected and the samples were re-analysed. The detection limit of the assay for corticosterone was 4.1 ng/mL, and concentrations are expressed as ng/g dry faeces.

Statistical analyses

Site, exclosure and microhabitat effects on soil compaction and on tree characteristics (isolation, canopy size, and height of resprouts under canopies) were analysed by means of General Linear Models (GLMs) on $\log_{10}$-transformed data. Tree characteristics associated to the presence/absence of mice during trapping sessions were analysed by means of logistic regression models, as most trees were occupied by only one individual. Categorical independent variables were year (winter 2010-2011 or winter 2011-2012), season (November or February), site, and exclosure (inside or outside), and continuous predictors canopy size, area covered by resprouts and tree isolation. We tested effects of categorical and continuous predictors and its two-way interactions on tree occupancy by mice as response variable by means of a forward stepwise procedure based on the Wald's statistic with p-to-enter and p-to-remove = 0.05 (Hosmer & Lemeshow 2000). Finally, we used GLMs to analyze factors explaining variation in FCM levels. Independent variables were site, trapping session, exclosure

(inside/outside), sex and breeding condition of individuals set as fixed factors. Body weight, local mice abundance, tree canopy and soil compaction were continuous covariates. Local mice abundance was estimated as the number of mice caught in the trees located in 40-m radius circles around trapping sites (Díaz et al. 2011; Muñoz & Bonal 2011). Original variables were log- or square-root transformed as needed to normalize distributions (Zar 1999). Results are given as mean ± standard error (SE). We used the SPSS 15.0 statistical software (SPSS Inc, Chicago, IL, U.S.A.).

RESULTS

Effects on vegetation variables and soil compaction

Local tree density did not differ inside an outside exclosures ($F_{1,168}$= 0.01, P = 0.946; 20.4 ± 1.5 trees/ha), and there were no exclosure x site effects ($F_{1,168}$ = 1.94, P = 0.166). Tree size (canopy cover) was not affected by ungulate exclosure ($F_{1,168}$ = 2.92, P > 0.090). Resprout height was three times shorter outside exclosures ($F_{1,168}$ = 12.75, P < 0.0001; 8.7 ± 0.2 *vs.* 26.8 ± 0.2 cm; backtransformed). The exclosure supported higher cover of resprouts around trees on average (1002.70 ± 83.65 cm^2) than outside (826.80 ± 76.80 cm^2), although differences were not significant ($F_{1,168}$ = 2.92, P > 0.090). Resprout height and cover were strongly correlated (r= 0.833, P < 0.0001), so that only cover was retained for further analyses. Tree isolation had a weak but significant positive effect on both (r= 0.217, P = 0.004 and r = 0.226, P = 0.003, respectively), and canopy size had a significant weak negative effect on resprout height (r = 0.226, P = 0.003). Exclosure effects were also highly significant on soil compaction ($F_{1,2382}$ = 61.65, P < 0.001), although microhabitat-dependent ($F_{1,2382}$ = 4.21, P = 0.040; exclosure x microhabitat interaction). Compaction was 2.7 times higher outside exclosures on average, and it was higher under trees than in the open grassland (1.4 *vs.* 1.2 times higher; Fig. 11). Exclosure effects on vegetation and soil traits did not differ among sites, as shown by non-significant site x exclosure interactions (details not shown).

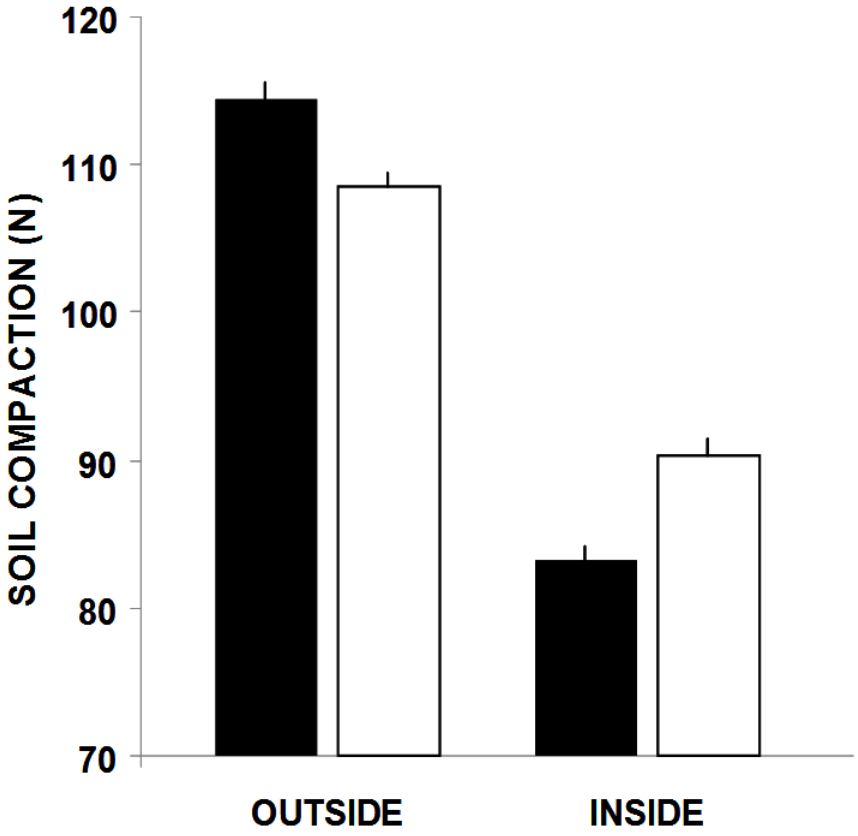


Figure 11. Mean (+SE) values of soil compaction outside and inside ungulate exclosures according to microhabitats. Filled bars: under oak canopies; open bars: open grassland

Habitat selection by mice

During the study we captured a total of 177 individual small mammals (recaptures were excluded), most of them (158; 89%) were Algerian mice (we also caught 18 wood mice and one common shrew *Crocidura russula*, that were not considered further). Abundance of Algerian mice was larger inside exclosures (Table 9), where mice occupied 60% of the trees as compared to 30% outside. Habitat selection was for smaller trees with more area covered by resprouts, and this selection was stronger outside than inside exclosures (Table 9 and Fig. 12). Resprout height did not influence tree occupancy. Significant stronger selection for less isolated trees in the eastern site and for larger trees when more isolated were also found (Table 9), but effect sizes for these two later interactions were almost zero as indicated by very low B values. Tree selection did not vary among years or seasonally, and the proportion of trees occupied by mice did not change significantly in time either.

Table 9. Results of the forward stepwise logistic regression (p-to-enter and p-to-remove = 0.05) testing whether presence of mice in trees was related to year, season, site, exclosure treatment (categorical preditors), canopy size, area covered by resprouts and tree isolation (continuos predictiors) and its two-order interactions

	Wald statistic	df	*P*	B	SE
Intercept	1.73	1	0.1885	0.46420	0.35302
Area of resprouts	39.42	1	0.0000	0.06597	0.01051
Canopy area	25.37	1	0.0000	-0.04032	0.00801
Exclosure (inside)	16.38	1	0.0001	0.83423	0.20615
Exclosure x resprouts	11.07	1	0.0009	0.03466	0.01042
Site x isolation	12.81	1	0.0003	-0.00087	0.00024
Canopy x isolation	8.25	1	0.0041	0.00002	0.00001

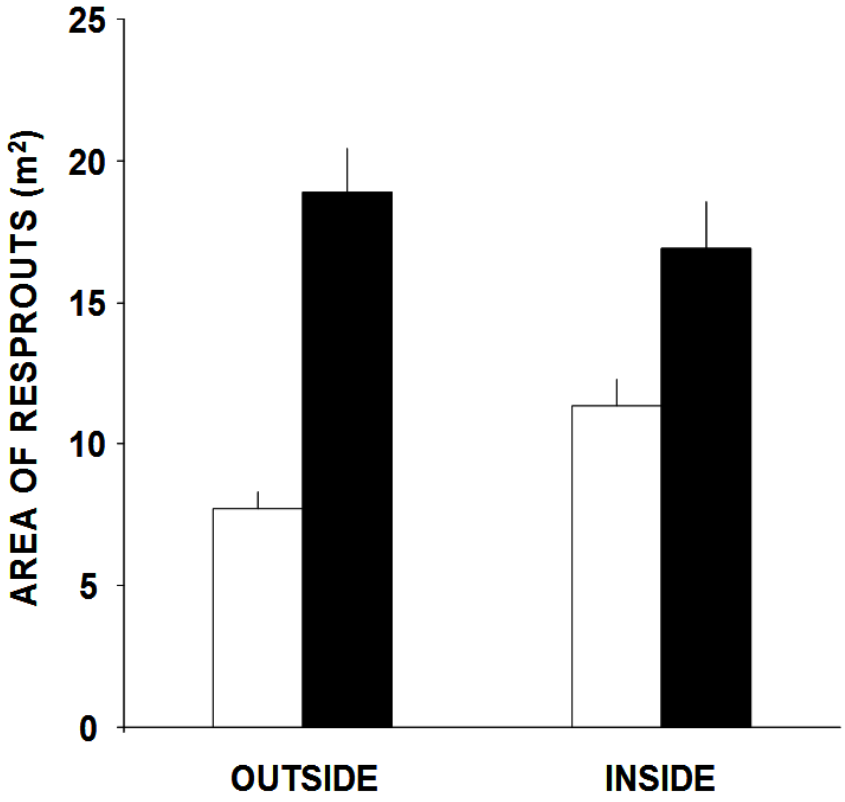


Figure 12. Mean (+SE) values of area under the tree canopy covered by resprouts for scattered tree occupied (filled bars) and not occupied (empty bars) by Algerian mice *Mus spretus* either outside or inside ungulate exclosures

Faecal Corticosterone Metabolites (FCM)

From the 158 Algerian mice captured during live trapping, fresh faecal samples to analyze FCM were available for 92 different individuals (Table 10). The influence of each factor on FCM levels is represented in Table 3. We did not find any evidence of direct effects of the zone, trapping sessions, breeding condition or body weight of individuals on FCM levels (Table 10). FCM values were influenced by the sex of individuals (females: 33392 ± 4060 ng/g dry faeces, males: 30095 ± 4524 ng/g dry faeces; $F_{1, 91} = 5.27$, $P = 0.026$). Part of the FCM variation was also explained by habitat characteristics. We found effects of canopy size and soil compaction on FCM levels. Individuals living under larger trees (with smaller cover of resprouts under them) and stronger soil compaction showed higher FCM levels (Fig. 13 A, B). The exclusion of ungulates also had an effect on FCM levels, individuals captured inside exclosures had higher FCM levels (inside: 33041 ± 3539 ng/g dry faeces, outside: 29225 ± 5488 ng/g dry faeces; $F_{1, 91} = 9.86$, $P = 0.003$). In addition, FCM levels were directly related to the local abundance of mice (Table 10). Individuals captured inside exclosures, where abundance of conspecifics was significantly higher, showed higher FCM levels (Fig. 14).

Table 10. Results of factorial ANOVA testing the effects of different environmental and individual factors on the variation of FCM in Algerian mice (*Mus spretus*)

Factor	**F**	**df**	***P***
Zone	1.485	1	0.229
Trapping sessions	0.826	3	0.486
Exclosure (Inside/Outside)	9.861	1	0.003
Sex	5.272	1	0.026
Breeding condition	2.183	1	0.147
Body weight of individuals	0.166	1	0.686
Local mice abundance	5.380	1	0.025
Tree canopy size	6.601	1	0.014
Soil compaction	7.018	1	0.011
Zone*Trapping sessions	2.536	2	0.090
Sex*Breeding condition	2.935	1	0.094
Zone*Exclosure*Sex	3.286	1	0.077

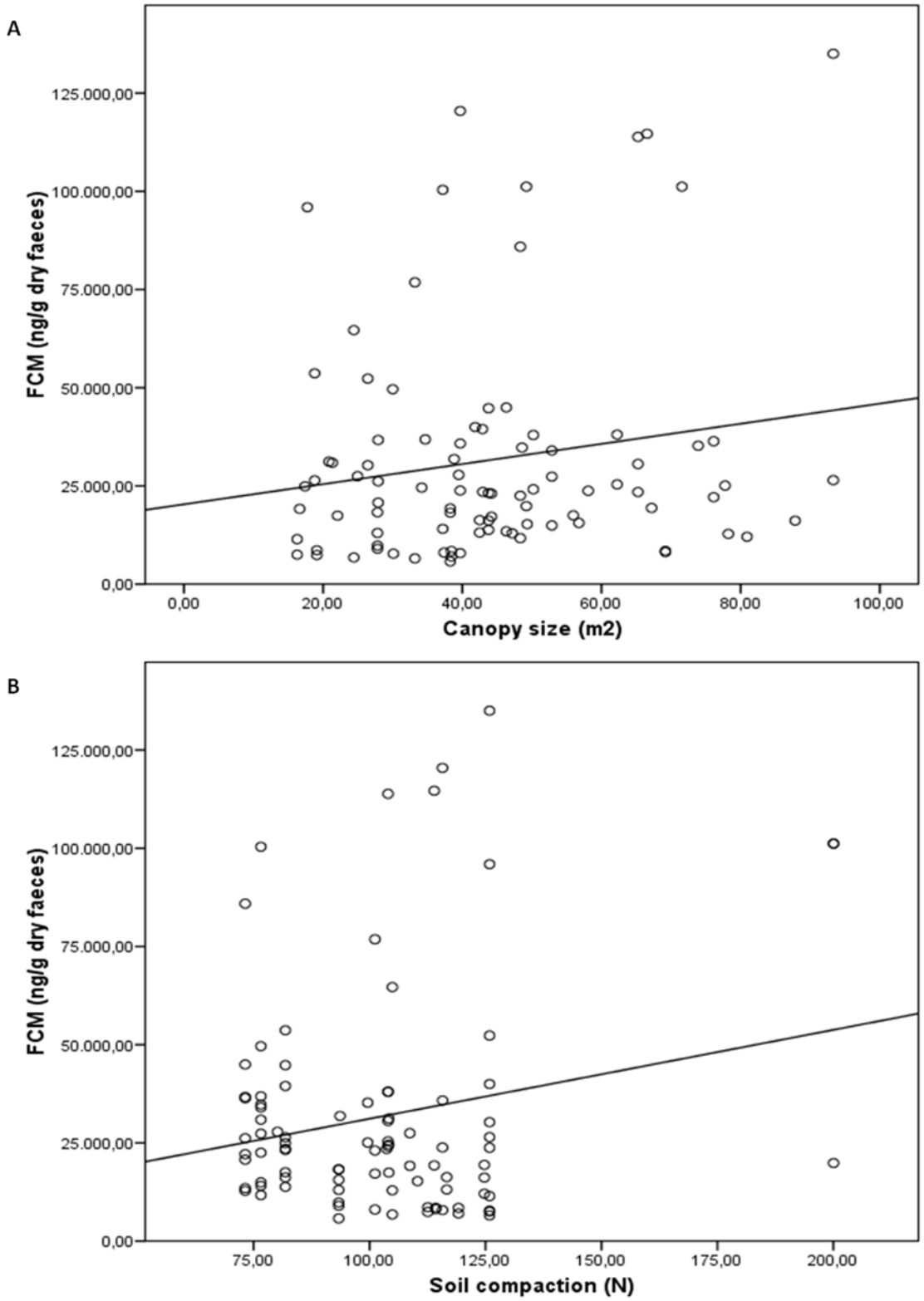


Figure 13. Relationship between concentrations of faecal corticosterone metabolites (FCM; ng/g dry faeces; mean ± SE) and canopy size (A) and soil compaction (B).

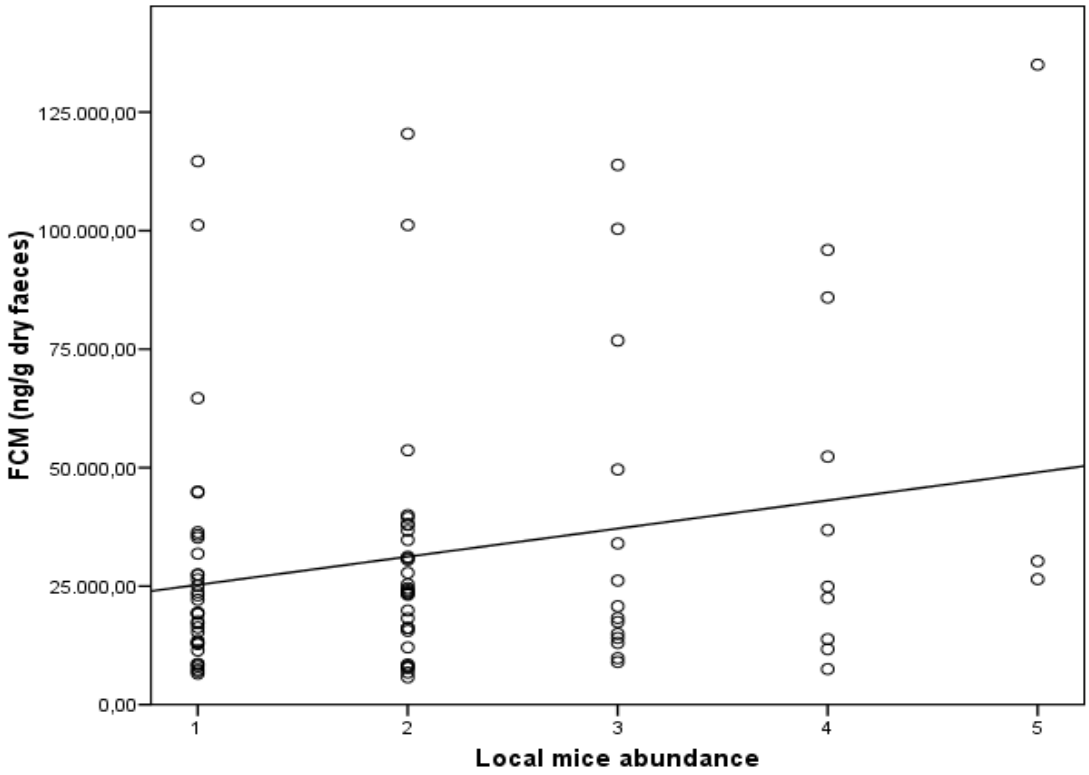


Figure 14. Relationship between concentrations of faecal corticosterone metabolites (FCM; ng/g dry faeces; mean ± SE) and local abundance of mice.

Interactions between factors were not statistically significant, but results indicate potential confounding effects of exclosure as it decreases soil compaction but increases local mice abundance. In fact, separate analyses of the effects of local mice abundance, tree size and soil compaction on FCM inside and outside exclosures (Table 11) showed that soil compaction influenced FCM levels outside buy not inside exclosures, and local mice abundance affected FCM inside exclosures but not outside them.

Table 11. Effects of soil compaction, tree canopy size and local mice abundance on FCM levels inside and outside exclosures.

	Exclosures			Grazed areas		
Factor	**F**	**df**	***P***	**F**	**df**	***P***
Local mice abundance	4.753	1	0.034	0.650	1	0.427
Tree canopy size	2.735	1	0.104	0.136	1	0.715
Soil compaction	0.185	1	0.669	4.643	1	0.040

DISCUSSION

Ungulate exclosure had mainly significant effects on soil compaction and resprout height, but weak effects on the area of resprouts as previously reported (e.g. McIntyre & Lavorel 2001; Rodríguez et al. 2003; Torre et al. 2007). Ungulate activity greatly increased soil compaction being significantly higher outside exclosure, specially under tree canopies, due to the trampling effect of ungulates that seemed to concentrate its foraging and resting activities under scattered oak canopies as compared to the open grassland (Treydte et al. 2010 and pers. obs.). Since Algerian mice potentially compete with ungulates for food resources, specially acorns, in some periods of the year (Díaz et al. 1993; Focardi et al. 2000; Muñoz & Bonal 2007) the absence of ungulates inside exclosures could cause an increase of the availability and/or quality of food resources (Smit et al. 2001; Schmidt et al. 2005). Better vegetation characteristics (i.e. greater and higher vegetation cover) together with a lesser compaction of soil which provide availability and easier construction and maintenance of burrows (Khidas & Hansell 1995; Hayward et al. 1997) could make exclosures a better habitat for small mammals. In our study system, vegetation, soil, and even mice (see below) responses to ungulate exclosure did not differ among two nearby sites excluded 2 ad 15 years before sampling; this lack of 'historical' effects may have been due to the slow recovery cycles of Mediterranean vegetation (e.g. Doblas-Miranda et al. 2015), that would have needed longer differences in time since exclosure to detect them, as well as to the short life cycles of Algerian mice (Palomo et al. 2009), that would have needed shorter times for detection.

The abundance of Algerian mice was strongly related to ungulates. Captures increased inside exclosures as a result of ungulates absence, coincident with previous studies (Torre et al. 2007; Muñoz et al. 2009). Mice occupied twice as many trees inside than outside exclosures. Tree occupation was stable, as indicated by lack of year or seasonal effects. Browsing effects on resprout height were strong, but unlikely to affect small mammals since resprout height did not influence tree selection by mice. It seems that mean height of browsed resprouts (ca. 10 cm) was still large enough to conceal mice against predators (Torre & Díaz 2004). However, selected trees had larger covers of protective resprouts, being this selection stronger outside exclosures. These results suggest that behavioral responses to habitat characteristics would have reduced direct

effects of ungulates (competition for food and/or trampling; (Smit et al. 2001; Schmidt et al. 2005; Torre et al. 2007) and indirect effects mediated by predation risk (Jensen et al. 2003; Torre 2004; Muñoz et al. 2009; Perea et al. 2011).

Inter-specific competition for food resources, physical disturbance and facilitation of small mammals' predation seem to be the most important interactions between wild ungulates and Algerian mice. Since environmental changes and disturbing factors can act as physiological stressors for natural populations (Wingfield et al. 1997) and the impact of ungulates on this rodent species is perceived as acceptable, consideration of possible physiological effects and consequences might be important in order to fully understand the impact of wild ungulates and habitat change on small mammal populations.

Our results showed sex of individuals having an important influence over FCM levels, with females showing significantly higher levels than males. This pattern has been reported in other wild rodent species (Touma et al. 2004; Navarro-Castilla et al. 2014ab) and this difference could be partly due to differences in the metabolism of glucocorticoids between both sexes (Touma et al. 2003). Higher FCM levels in females than in males perhaps could be due to the influence of female sex steroids on the synthesis and release of GCs from the adrenal cortex (Carey et al. 1995).

The scattered oaks in the grazed plots are important sources of food being frequently visited by ungulates and probably intensifying their impact on rodents (Treydte et al. 2010). Canopy size resulted as an important factor influencing FCM levels. Canopy size was inversely related to resprout availability, so the observed increase in FCM levels could be due to a decrease in protection by resprouts against predators (Preston 1990; Sheffield et al. 2001; Orrock et al. 2004) or, more unlikely, by larger risk under larger trees maybe due to attraction to predators, competitors, or both. In addition to grazing, other ungulate physical disturbances which also damage burrows systems, such as direct trampling (Hayward et al. 1997; Daniel et al. 2002; Torre et al. 2007) and rooting or predation by wild boar (Focardi et al. 2000; Schley & Roper 2003; Herrero et al. 2006) could be acting as potential stressors for Algerian mice. Soil compaction cause caverns or changes in water infiltration being directly related to the availability and maintenance of burrows (Khidas & Hansell 1995; Hayward et al. 1997). Since small rodents like the Algerian mouse depends on underground burrows (Khidas

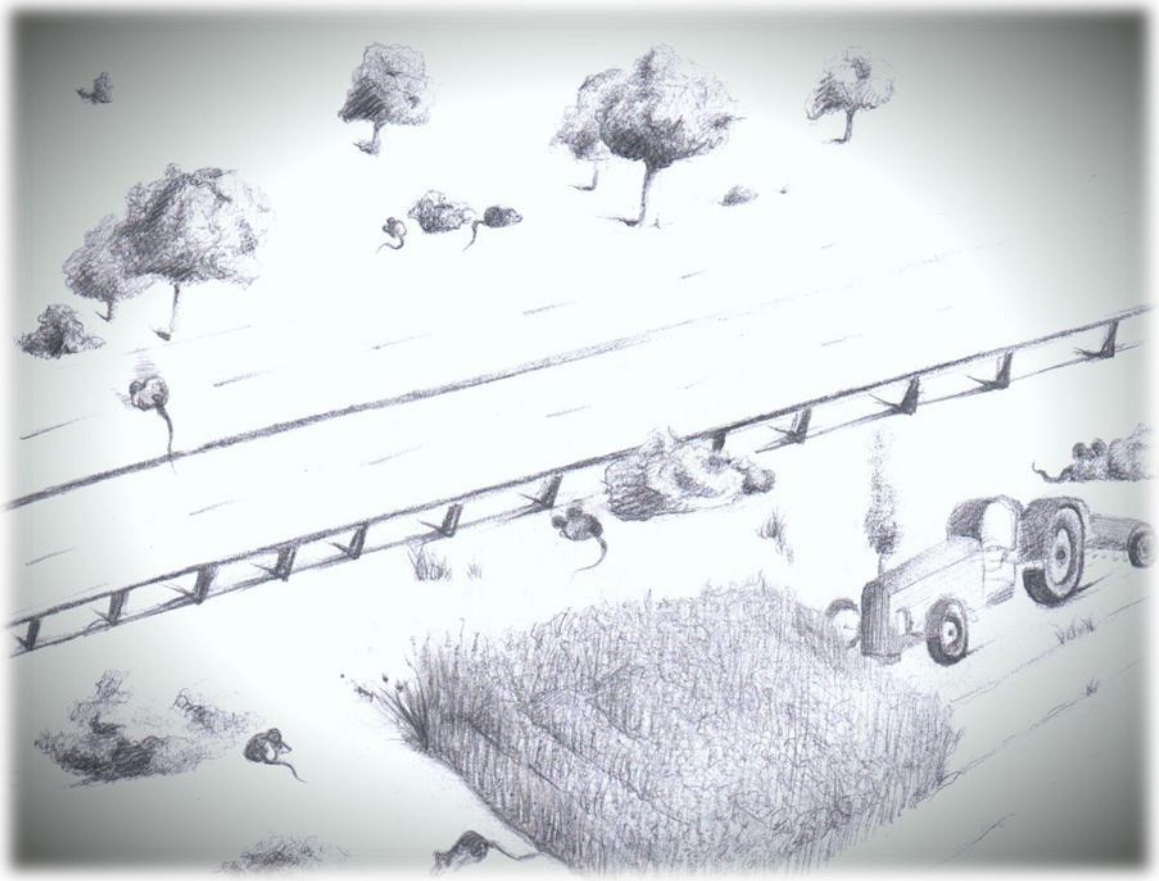
& Hansell 1995), the increase in FCM in more compacted sites detected in trampled grazed plots could be a response of this species to this indirect effect of ungulates on mice (Torre et al., 2007; Muñoz et al., 2009). The lack of local soil compaction effects on FCM inside exclosures, where soils are less trampled and looser, could be interpreted in the same vein.

Habitat characteristics and their influence on FCM levels would lead to predict higher stress-mediated FCM levels outside exclosures as a response to the effects of ungulates. Nevertheless, we found somewhat opposite patterns since FCM levels were in fact higher inside, so it seems that potential positive effects of increased food availability and reduced trampling inside exclosures were counter-balanced by other ungulate-mediated effects on individual mice. Thus, local mice abundance appeared as an important factor positively correlated with FCM levels. Studies on the relationships between population density and adrenal activity have yielded mixed results to date. For some species, increased population densities resulted in an increase in adrenal activity (Goymann & Wingfield 2004; Raouf et al. 2006), while for other species this relationship was not well supported (Seal et al. 1983; Bradley et al. 1988; Armitage 1991; Harper & Austad 2004). In this study, local increases of Algerian mice abundance were associated with higher levels of FCM inside exclosures, were density was higher, but not outside, where population density was much lower. This finding suggests that social stress may be greater under higher population densities because of increased social contact (e.g. intraspecific competition, increasing aggression rates; Carobrez et al. 2002) and decreasing the availability of resources (food, refuge and/or partners) which has an important impact on small rodent populations (Lemen & Clausen 1984; Byrom et al. 2000). Therefore, the high local densities promoted by ungulate exclosure could have lead to social instability causing increased FCM levels in the Algerian mouse, as reported for several species (Boonstra & Boag 1992; Rogovin et al. 2003; Harper & Austad 2004; Navarro-Castilla et al. 2014a). This increase in FCM levels could be a positive physiological response to cope with increased social interactions associated to high local densities since a recent study on a wild rodent (Dantzer et al. 2013) demonstrated the benefits of increased adrenocortical activity in case of high population density.

To the best of our knowledge this is the first report studying wild ungulates effects on physiological stress responses in mammals being a significant contribution to the knowledge of environmental factors affecting glucocorticoids levels in wild animals. Since hormone levels in wild ranging animals can be influenced by a wide range of environmental factors and, the adrenocortical responses evoked to cope with a stressor reflect individual variation (Moberg 1985; Moberg 2000), additional research is needed in order to better understand how animal populations react and cope with natural disturbances and alterations of the surrounding environment.

CHAPTER 3

HUMAN DISTURBANCES



PAPER V

Navarro-Castilla, Á., Barja, I., Olea, P.P., Piñeiro, A., Mateo-Tomás, P., Silván, G. & Illera, J.C. (2014). Are degraded habitats from agricultural crops associated with elevated faecal glucocorticoids in a wild population of common vole (*Microtus arvalis*)? *Mammalian Biology* 79: 36-49.

PAPER VI

Navarro-Castilla, Á., Mata, C., Ruiz-Capillas, P., Palme, R., Malo, J.E. & Barja, I. (2014). Are motorways potential stressors of roadside wood mice (*Apodemus sylvaticus*) populations? *PLoS One* 9:e91942

PAPER V

ARE DEGRADED HABITATS FROM AGRICULTURAL CROPS ASSOCIATED WITH ELEVATED FAECAL GLUCOCORTICOIDS IN A WILD POPULATION OF COMMON VOLE (*Microtus arvalis*)?

ABSTRACT

The severe impact of agriculture on species' abundance and diversity is widely recognized. However, its effects on the physiology of wild animal populations are poorly known. We analyzed faecal glucocorticoids levels in wild common voles (*Microtus arvalis*) living in a farmland landscape to test whether living in degraded habitats, such as crops, is correlated with increased glucocorticoids. Other factors such as sex, reproductive status, and population density were also considered. We captured voles with Sherman traps in crops and in their field margins which were comprised of semi-natural vegetation. We collected fresh faecal samples from captured individuals and quantified their levels of faecal corticosterone metabolites (FCM) in the laboratory. The quantification of FCM concentrations was performed by competitive enzyme immunoassay. Individuals captured within the crops had higher levels of FCM than those in field margins; females and breeding individuals exhibited higher FCM levels. In addition, FCM concentrations positively correlated with abundance of voles. Our results suggest that degraded habitats in agricultural landscapes are associated with increased glucocorticoid levels on common voles likely caused by a higher disturbance from agricultural practices and a lesser vegetation cover in crops compared with field margins.

Keywords: anthropogenic disturbance, faecal corticosterone metabolites, field margins, managed landscapes, small mammals

INTRODUCTION

Agriculture is considered to be a major cause of global change and biodiversity loss. While it is widely known that agriculture affects the composition and abundance of species (Tucker et al. 1994; Krebs et al. 1999; Donald et al. 2001; Robinson & Sutherland 2002; Benton et al. 2003; Wickramasinghe et al. 2003; Brotons et al. 2004; Ursua et al. 2005), its effects on other more subtle components such as physiological changes in wild animals are largely unknown.

Agricultural activities are human-made interventions that involve the modification of the original natural ecosystems which are replaced by a series of degraded habitats that are exposed to continuous modifications such as tilling, harvesting or crop rotation (Ammann 2004). Since habitat change may act as stressor in wild animals (Wingfield et al. 1997), the environmental changes and disturbing factors caused by agriculture may act as potential stressors for natural populations. Among the responses to physiological stressors, there is an increase in the synthesis and secretion of glucocorticoids (GC) (Melmed & Kleinberg 2003; Stewart 2003). Thus, GC levels have been used as physiological indicators of animal stress in several species (Möstl & Palme 2002; Stewart 2003) including small mammals (Harper & Austad 2001; Hayssen et al. 2002; Good et al. 2003; Fletcher & Boonstra 2006; Ylönen et al. 2006; Götz & Stefanski 2007). Although short-term GC secretion is related to animal adaptive responses (Wingfield & Romero 2001), chronically elevated and prolonged high GC levels can be harmful to individuals and produce an array of pathological consequences such as reproductive disruption, suppression of the immune function, inhibition of growth, survival and decline in fitness (Munck et al. 1984; Sapolsky 1992; Sapolsky et al. 2000; Sapolsky 2002; Stewart 2003; Romero 2004).

Assessing GC concentrations in blood plasma samples has traditionally been considered as one of the accepted indicators of quantitative measurements of physiological stress conditions (Broom & Johnson 1993). However, this technique is an invasive method and requires animal capture, handling, sedation and/or transport and puncture (Graham & Brown 1996; Brown & Wildt 1997; Dehnhard et al. 2001; von der Ohe et al. 2004; Young et al. 2004) which can cause an induction of a stress response during sampling procedures (Cook et al. 2000; Place & Kenagy 2000). Alternatively, methods for non-invasive measurement of faecal glucocorticoid metabolite (FG) levels

have been widely used. Faecal samples for FG analysis are relatively easy to collect without disturbing animals (Monfort et al. 1998) and these are suitable techniques to evaluate stress physiology response and endocrine status in wildlife (Monfort et al. 1997; Palme et al. 1998; Goymann 2005; Touma & Palme 2005; Barja et al. 2012). This non-invasive method has been applied for investigating adrenocortical activity during exposure to stressful stimuli in an increasing number of mammalian species (e.g. mouse and vole: Harper & Austad 2000; Ylönen et al. 2006; elk: Millspaugh et al. 2001; bear: von der Ohe et al. 2004; European pine marten: Barja et al. 2007; wolf: Barja et al. 2008; wild rabbit: Monclús et al. 2009; spotted hyena: van Meter et al. 2009; wildcat: Piñeiro et al. 2012) . These studies have evaluated the stress response under different natural events (Wingfield et al. 1997; Boonstra et al. 1998), as well as the physiological response due to human influence (tourism, winter sports, snowmobile activity) in natural systems (Millspaugh et al. 2001; Creel et al. 2002; Barja et al. 2007; Thiel et al. 2011; Piñeiro et al. 2012; Zwijacz-Kozica et al. 2013). Yet, the physiological responses of wild mammal populations in highly managed (agricultural) landscapes are scarcely known.

OBJECTIVES

The aim of the present study was to test the hypothesis that living in degraded habitats, resulting from the creation of agricultural crops, is correlated with an increase in faecal corticosterone metabolite (FCM) levels in a small mammal, the common vole. Voles of genus *Microtus* are rodents famous for their population fluctuations which sometimes can cause economic losses due to crop damage (Olea et al. 2009). They typically inhabit grassland, fallows and fields and they live in burrows, ca. 50 cm deep and breed mainly from April to November (Jacob 2000). In addition, they are an important food source for predatory birds and mammals (Halle 1993), so the state of vole populations is expected to have not only economic but also noticeable ecological impacts. We studied FCM levels in common voles living within crops and in seminatural habitat remains (i.e. field margins) in an agricultural landscape. Compared with crops, seminatural field margins are far less or not manipulated (e.g. irrigation, agricultural management practices, use of pesticides) and present higher vegetation cover, so may be considered as a better quality habitat in terms of disturbance and predation risk (Jacob & Brown 2000). Therefore, if agricultural management and degraded habitat in crops acts like

stressful factors, FCM levels should vary in relation to the type of habitat. We would expect higher FCM levels in those individuals living within crops, and therefore subjected to a major degree of environmental disturbance and degradation and with a higher predation risk perceived (Jacob & Brown 2000), than in the seminatural habitat of field margins.

MATERIAL AND METHODS

Study area

The fieldwork was conducted in the southeast of the León province, northwest of Spain (centred on 42°33N, 5°31W) at an altitude of approximately 800 m a.s.l. within the Mediterranean climatic region. The study area covers approximately an extension of 750 km^2 of farmland with crops mainly comprised of dry cereal yields (wheat and barley).

Nine plots of 500x500 m consisting of agricultural crops were randomly arranged within the study area. Within each plot, two fields were selected for live-trapping. From the total number of selected fields (i.e. 18), four fields were discarded because of logistical problems or because fresh faecal samples were not available, resulting in a total of 14 sampled fields.

Animal trapping and data collection in the field

Trapping was conducted in July 2007, before the harvest of cereal, during a peak of abundance of voles (Olea et al. 2009). For the capture of voles we used Shermann® live traps. In each field we placed 14 traps in two parallel lines of 7 traps, one trap line within the crop and the other one in the field margin. The two trap lines were 5 m apart, and within the two trap lines, each trap was separated from each other by 10 m, covering an area of 1050 m^2 per field. This area potentially included the entire home range of a common vole [sizes of home range: 125 m^2 (95% of spatial use: 31 m^2) in wild flower strips (Briner et al. 2005), 145 m^2 in alfalfa and pasture (Mackin-Rogalska 1981) and 350 m^2 in winter corn (Mackin-Rogalska 1981)]. Taking into account general home ranges for this species, proximity between the trap lines in field margins and within crops made the access to both habitats by the same individual perfectly possible. However, voles in field margins are not expected to move into adjacent fields if habitat quality and food abundance are sufficient (Briner et al. 2005). In addition, voles did not

expand their home ranges from field margins into the surrounding crops at high population densities (650 individuals/ha; Briner et al., 2005), even when there were highly palatable food in the adjacent crop (Balmelli et al. 1999). Thus, we expected that voles from field margins in the study area did not move into de adjacent crop. Traps were hidden under vegetation cover to protect animals from adverse weather conditions and to avoid detection by predators. Traps were baited with wheat collected from crops of the study area. Traps were set on three consecutive nights, and the total effort was of 588 traps-night. We reviewed the traps every 8-10 hours, at dawn and dusk, to minimize the time that animals were kept.

For each capture, habitat type (i.e. crop / field margin), body weight, sex (i.e. female / male), and reproductive condition of individuals (i.e. breeding / non-breeding) were recorded. To determine the sex it was considered that the distance between the clitoris and anus in females is smaller than the distance between the penis and anus in males (Gurnell & Flowerdew 1994). In breeding adult males the testes enlarge quite markedly and usually descend into the scrotal sac. In breeding adult females, nipples on the abdomen and thorax are noticeable when females start suckling the litter and they are still distinguishable after finishing lactation. Also, in mature females the vaginal membrane perforates prior to the first oestrus (Gurnell & Flowerdew 1994).

All captured individuals were handled as fast as possible to reduce disturbance, and they were released in the same place of capture. Traps were cleaned with clean water to avoid the mixing of different samples and then reactivated. Captured animals were marked in non-conspicuous areas with harmless watery paints (red food colouring: Ponceau-4R E124) to identify possible recaptures and remove them from the sample in order to achieve data independence. Manipulations of animals were done in compliance with the European Communities Council Directive 86/609/EEC for animal experiments and were carried out under the permit of the Dirección General del Medio Natural de la Consejería de Medio Ambiente de la Junta de Castilla y León number EP/LE/281/2007.

As high densities usually generate a major degree of intraspecific competition, increasing aggression rates and social instability (Carobrez et al. 2002; Rogovin et al. 2003), we estimated common vole abundance to account for its influence on faecal corticosterone metabolite (FCM) levels. As a proxy of abundance at each site we

considered the number of voles captured during the live-trapping sessions excluding recaptured ones.

Faecal sample collection and enzyme immunoassay (EIA)

During the live-trapping no more than one individual per trap was captured simultaneously. We collected a minimum of 4 fresh faecal pellets (i.e. with a soft texture and not dried) found in traps where a common vole had been captured, but we rejected those faecal samples from traps where urine was detected in order to avoid cross contamination between urine and faeces. We only collected fresh samples to avoid the effects of environmental conditions such as heat or proliferation of microorganisms what could affect to faecal glucocorticoid metabolite levels as a result of their own metabolism (Washburn & Millspaugh 2002; Millspaugh et al. 2003). Touma et al. (2004) found that corticosterone concentrations are observed in laboratory mice faeces up to 10 h after the ACTH injection (median: 10 h, range: 8–12 h). Thus, we reviewed traps within 10 h after activation to avoid a possible effect of the capture in glucocorticoid levels, although Harper & Austad (2001) found that there was not any effect of trapping on faecal glucocorticoid levels in wild red-backed voles (*Myodes gapperi*) after 12 h of confinement in traps. To avoid the influence of circadian rhythm in excretion patterns (Touma et al. 2003) only fresh samples collected during the early morning checking (i.e. 7:00 to 9:00 am) were considered for the study. All faecal samples were collected in sterile tubes appropriately labeled and kept on ice during less than two hours of transport from field to laboratory. In the laboratory, samples were stored in a freezer at -20º C until be analyzed.

Frozen faecal samples were dried at 50 ºC in a laboratory heater (Selecta, model CONTERM 2000208) until constant weight. The extraction of glucocorticoid metabolites from the faeces was performed according to the method described by (Touma et al. 2003). Briefly, faecal samples were homogenized with mortar and pestle and 0.05 g were weighed and mixed with 1 ml of 80% methanol in an eppendorf tube. Samples were shaken for 30 minutes on a multivortex and then centrifuged for 15 minutes at 2500 x *g*. Supernatants were obtained diluted 1:10 with assay buffer and maintained at -20ºC until analysis.

For the analysis of corticosterone metabolite concentrations in the faeces we used an enzyme immunoassay (EIA). This technique (EIA) has been used in several

mammal species (e.g. European pine marten: Barja et al. 2007; rats and mice: Lepschy et al. 2007; Nováková et al. 2008; elk: Creel et al. 2009; European badger: Barja et al. 2012, wildcat: Piñeiro et al. 2012). The EIA was done in the laboratory of Endocrinology of the Veterinary Faculty (Complutense University, Madrid) for plasma of rodents by Silvan et al. (2007) and validated specifically for common vole faeces following the methods described by Bamberg et al. (2001) and Morrow et al. (2002). Polyclonal antibody (CT1098) was raised in our laboratory in rabbits against corticosterone 3-CMO:BSA (Steraloids Inc., Newport, USA). Cross reactivity of the corticosterone antibody CT1098 was: Corticosterone: 100%; Aldosterone: 10.5%; Prednisolone: 5.71%; Prednisone: 8.9%; Cortisone: 10.8%; Cortisol: 6.4%; 11-Deoxycorticosterone: 14.31%; 21-Deoxycorticosterone: 5.31%; Progesterone, estradiol, testosterone and estrone sulphate <0.1%. The range of standard curve was from 0.1 to 0.000 pg/100 µl, and the detection limit of the assay was 3 ng corticosterone metabolites/g dry faeces. Parallelism was performed by comparing serial dilutions of pooled faecal extracts and the standard curve demonstrating that binding inhibition curves of serially diluted pools of faecal extracts (1:1 to 1:50 fold dilutions) were parallel to the standard curve (ANCOVA: $F_{1, 11} = 0.141$, $P = 0.714$; Fig. 15).

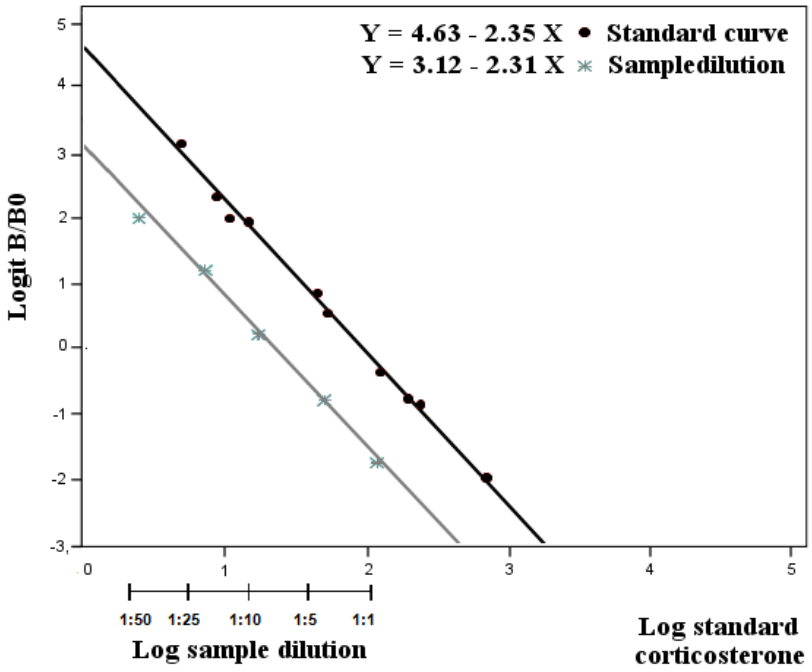


Figure 15. Parallelism test performed by comparing serial dilutions of pooled faecal extracts (1:1 to 1:50 fold dilutions) and the standard corticosterone curve. Binding inhibition curves of serially diluted pools of faecal extracts were parallel to the standard curve.

The addition of exogenous amounts of corticosterone (0.1; 1.0; 10.0 µg corticosterone/g faeces) added to pooled faecal samples with high (30.5 µg corticosterone/g faeces) and low (15.3 µg corticosterone/g faeces) faecal corticosterone metabolite concentrations showed a mean recovery of 95 %. Intra-assay coefficient of variation (CV) was calculated by assaying ten times pools of faecal samples within an assay. Inter-assay CV was calculated by assaying the same pools of faecal samples in ten consecutive assays. Intra- and inter-assay CV were 6.6 % and 9.9 % with low concentrations, and 5.9 % and 8.9 % at high FCM concentrations. FCM concentrations are expressed as µg/g of dry faeces.

Statistical analysis

We used General Linear Mixed Models (GLMMs) to analyze factors influencing FCM levels. The response variable, i.e. concentrations of FCM, was $\log_{10}$ transformed to reach normal distribution. We used Gaussian error mixed-effects models fit by maximum likelihood (ML) to compare AIC of models, and restricted maximum likelihood (REML) for estimation of parameters in the best model obtained (see below). Field and field nested within plot were fitted as random effects. This random structure (i.e. "1|Plot /Field") allows us account for: i) possible dependence (pseudoreplicates) among voles within same fields, ii) similarity of fields within the same quadrat and iii) spatial autocorrelation (Crawley 2007; Olea 2009). The fixed effects were sex (male / female), breeding condition (breeding / non-breeding), body weight, habitat type (crop / field margin) and abundance of common vole per field.

Best models were selected using a multimodel selection procedure (Burnham & Anderson 2002). We performed all possible model permutations with all the explanatory variables and the resulting models were ranked according to their AICc (i.e. corrected Akaike Information Criterion) value (Burnham & Anderson 2002) and the Akaike weight of each model (ω_m). The Akaike weight of each model was estimated following procedures by Burnham & Anderson (2002). We constructed a 95% confidence set of models by starting with the highest Akaike weight and adding the model with the next highest weight until the cumulative sum of weights exceeded 0.95 (Burnham & Anderson 2002). To find the relative contribution of each variable we also calculated their Akaike weights (Burnham & Anderson 2002; Mateo-Tomás & Olea 2009; Olea 2009). The Akaike weight for each variable (ω_i) was calculated by

summing, from the set of models within the 95% probability, the weights of those containing that variable. The variables with the highest weight (i.e. $\Sigma\omega_m = 0.95$) being more important relative to the others.

Because the sample size was relatively small (N = 60 voles), we kept the number of candidate models low and therefore tested quadratic response and interactions apart of the multimodel selection procedure described above. We tested for possible quadratic response (Busch & Hayward 2009) of the continuous variable abundance of voles on FCM by comparing univariate models with the same variable included linearly and non-linearly. Models with non-linear response were no better (in terms of AICc) than those with linear variables, so only linear variables were considered for multimodel selection. Two-way interactions with biological sense were also tested (i.e. sex*habitat type, sex*breeding condition, breeding condition*habitat type, abundance of voles*habitat type, abundance of voles*sex, abundance of voles* breeding condition, body weight*sex, body weight*habitat type, body weight*breeding condition). Models with and without the interaction were compared. Finally, we looked for whether the best models (within 2 AICc units from the top-ranked model) improved in terms of AIC with interactions fitted.

The best models (ΔAICc < 2, i.e. within two AICc units from the model with the lowest AICc) were investigated for goodness of model fit (Nakagawa & Schielzeth 2012) and validity of assumptions (normality and homogeneity of variances of residuals) using diagnostic plots. We used the nlme package (Pinheiro et al. 2012) for fitting GLMMs in the R statistical software (R version 3.0.1; (R Development Core Team 2013)). We used the REML method to fit the best AIC-based model (see above) and estimate from this model the coefficients and *p*-values of fixed factors as well as goodness of fit. This best model fulfilled assumptions of normality and homogeneity of variances of their residuals. Data are given as mean ± standard error (SE).

RESULTS

A total of 121 common voles were captured during live trapping and fresh faecal samples for analyzing faecal corticosterone metabolites (FCM) were available from 60 individuals (Table 12). The best models (ΔAICc < 2) explaining the variation in FCM levels included together four variables: habitat type, breeding condition, sex, and abundance of common vole ($\omega_m = 0.60$, Table 13). The best model (Table 13) explained

49% of the variance in faecal corticosterone. The four fixed effects, included together in the best model, explained 34% of the variance in faecal corticosterone.

Table 12. Data of total individuals captured and from the individuals which FCM were analyzed in relation to habitat type, sex and breeding condition. NB: non breeding, B: breeding.

Habitat	Captures	Recaptures	Abundance (individuals/ 100 traps-night)	Mean body weight (g) (± SE)	Number of total individuals captured (N = 121)				Number of individuals whose FCM were analyzed (N = 60)			
					Males (N = 52)		Females (N = 69)		Males (N = 19)		Females (N = 41)	
					NB	B	NB	B	NB	B	NB	B
Field margins	70	0	24	28.18 ± 1.29	27	5	18	20	10	1	8	14
Crops	51	4	17	26.15 ± 1.29	15	5	17	14	6	2	12	7
Total	**121**	**4**	**41**	**27.33 ± 0.93**	**42**	**10**	**35**	**34**	**16**	**3**	**20**	**21**

Table 13. Ranking of the models within the 95% confidence set ($\Sigma\omega_m$ = 95%) explaining the variation in faecal corticosterone levels. The null model is also included. The table shows number of parameters (K), AICc differences (Δ_i), Akaike weights (ω_m), and the rank of the models. HT: habitat type, BC: breeding condition, SX: sex, AB: common vole abundance, BW: body weight.

Model	K	ΔAICc	ω	Rank
HT+BC+SX+AB	8	0.00	0.302	1
HT+BC+SX	7	1.03	0.181	2
HT+BC	6	1.98	0.113	3
HT+BC+SX+AB+BW	9	2.58	0.083	4
HT+BC+AB	7	3.15	0.063	5
HT+SX+AB	7	3.30	0.059	6
HT+BC+SX+BW	8	3.41	0.055	7
HT+BC+BW	7	4.06	0.040	8
HT+BC+AB+BW	8	5.26	0.022	9
HT+SX+AB+BW	8	5.35	0.020	10
HT+SX	6	5.96	0.015	11
Null model	4	16.81	0.000	32

The relative importance of the variables included in the best models (Fig. 16), showed that habitat type was the most important variable explaining variations in FCM concentrations ($\omega_i = 1$, Fig. 16).

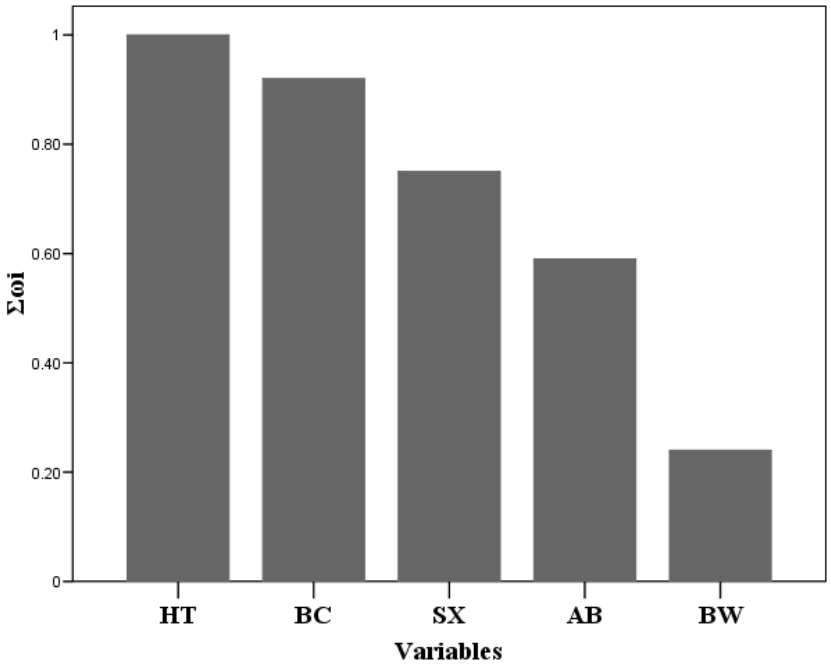


Figure 16. Relative contribution of each variable in the models within 95% confidence set ($\Sigma\omega_m$ = 95%) explaining the variation in faecal corticosterone metabolite levels according to the sum of its Akaike weights ($\Sigma\omega_i$). HT: habitat type, BC breeding condition, SX: sex, AB: common vole abundance, BW: body weight.

FCM levels were significantly higher in individuals captured within crops (26.1 ± 4.4 μg/g dry faeces) than individuals captured in field margins (14.5 ± 2.8 μg/g dry faeces) ($F_{1,\ 36}$ = 11.77, P = 0.0015; Fig. 17A). Breeding condition and sex also influenced variations in the concentrations of FCM in common voles ($\omega_i = 0.92$ and $\omega_i = 0.75$ respectively, Fig. 16). We found that FCM levels were higher in females (23.5 ± 3.8 μg/g dry faeces) than in males (14.6 ± 2.8 μg/g dry faeces) ($F_{1,\ 36} = 4.43$, P = 0.043; Fig. 17B). Breeding individuals exhibited higher FCM levels (31.1 ± 6.1 μg/g dry faeces) compared to the non-breeding ones (14.3 ± 1.9 μg/g dry faeces) ($F_{1,\ 36} = 5.80$, P = 0.021; Fig. 17C). Differences due to breeding condition were not significant for males (breeding: 22.5 ± 1.6 μg/g dry faeces, non-breeding: 13.5 ± 3.1 μg/g dry faeces; $F_{1,\ 4}$ = 1.52, P = 0.28; probably because of low sample size with only 3 breeding males) and significant for females (breeding: 32.1 ± 6.7 μg/g dry faeces, non-breeding: 15.2 ± 2.7 μg/g dry faeces; $F_{1,\ 20} = 6.30$, P = 0.021).

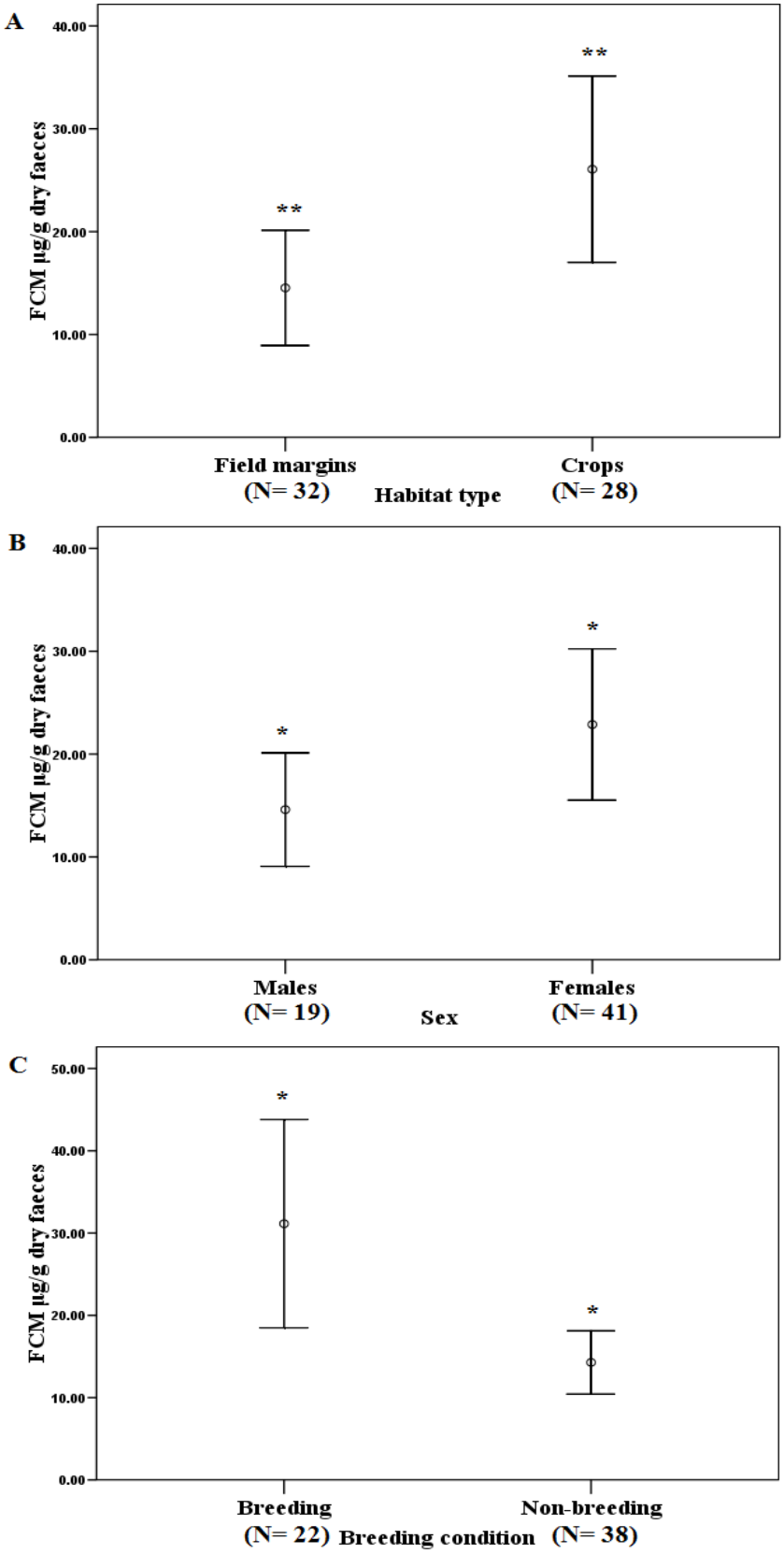


Figure 17. Comparison of faecal corticosterone metabolite levels (µg/g dry faeces) (mean ± SE) in relation to habitat type (A), sex (B) and breeding condition (C). Number of individuals analyzed for each category (N) is included and asterisks indicate significant differences between the analyzed groups (* $p < 0.05$, ** $p < 0.01$).

The abundance of common vole explained part of the FCM variation, but had a minor relative contribution to the models (ω_i = 0.59, Fig. 16). Individuals captured in fields with higher abundance of conspecifics showed higher FCM levels ($F_{1, 36}$ = 4.87, P = 0.034; Fig. 18). Body weight was not included in the best models and there was a very weak effect of this variable on vole's FCM (ω_i = 0.24, Fig. 16). Interactions were neither significant nor improved (i.e. reducing AICc) the best models when fitted into them.

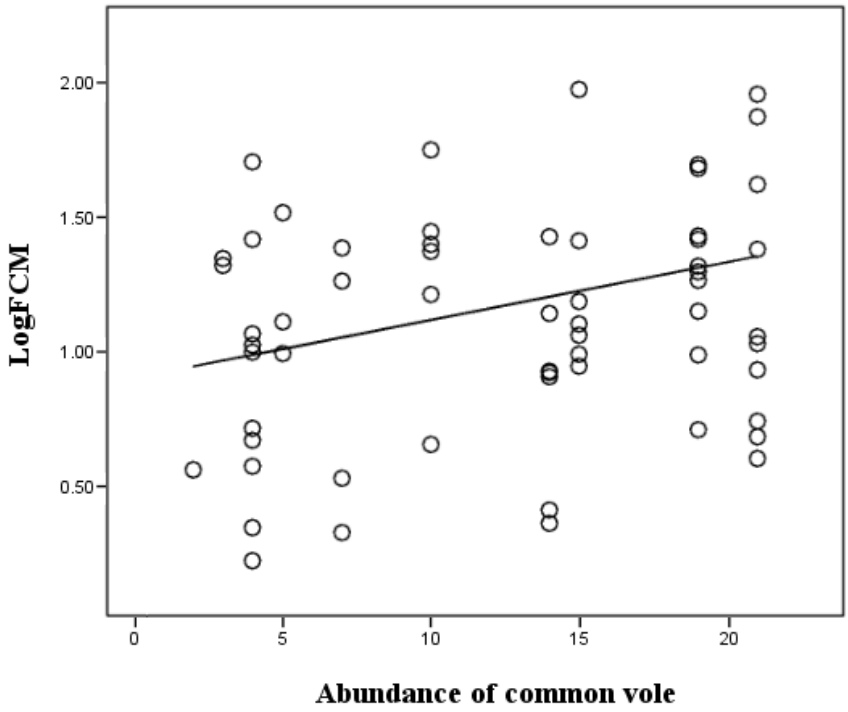


Figure 18. Plot of log-transformed concentrations of faecal corticosterone metabolites (FCM, μg/g dry faeces) of each individual vole according to the abundance of common voles registered in each field. The regression line is drawn from the coefficients of the best GLMM model (B_0 = 0.90; slope = 0.022, t_{36} = 2.21, P = 0.034); their residuals do not deviate from normality (Shapiro-Wilk normality test; W = 0.97, P = 0.22).

DISCUSSION

Our results showed that habitat type (field margin vs crop) was the most important variable explaining variations in FCM concentrations. This result matches with our prediction that voles living in a habitat subjected to a major degree of environmental disturbance would show higher FCM levels. Habitat complexity seems to be an important factor for small mammal communities. It has been reported that reduction in vegetation cover exposes small mammals to increased predation risk (Preston 1990;

Sheffield et al. 2001; Orrock et al. 2004), inducing behavioural changes in common voles (Jacob & Brown 2000). While agro-ecosystems with vegetation management provide habitats unattractive to voles (Sullivan & Hogue 1987), organic areas, such as abandoned fields or wildflower strips enhance food and cover for voles (Brown 1999; Briner et al. 2005; Heroldová et al. 2005). In our study area, seminatural field margins are expected to be a better quality habitat for voles than crops since these margins are much less managed (i.e. less pesticides, no ploughing) and provide more vegetation cover and thus a lower perceived risk of predation (Jacob & Brown 2000). We have no data about the predation rates of voles in field margins and crops in our study area, but Arlettaz et al. (2010) found that radiotracked Barn Owls in Switzerland preferred to forage in cereal fields and grassland rather than in field margins (wildflower strips) where there were more abundance of voles. These authors suggested that the dense vegetation cover of wildflower strips may be preventing access to prey for foraging owls. So, the lesser disturbance and higher protection from predators (i.e. more vegetation cover) could explain the lower FCM values that we found in voles captured in seminatural field margins compared with those captured within crops. Alternatively, differences due to social rank might also explain this pattern (Louch & Higginbotham 1967). Dominant individuals may occupy the high quality habitat (i.e. field margin) pushing the subordinates individuals to the stressful, lower quality habitat (i.e. crop).

Sex and breeding condition relationship with glucocorticoids can be variable, context dependent or even non-existing (Harper & Austad 2004; Schradin 2008; Busch & Hayward 2009). In our study, females and breeding individuals (both males and females) were those who showed higher FCM levels. Sex differences in FCM levels are supposed to be partly due to differences in the metabolism of glucocorticoids between both sexes (Touma et al. 2003; Goymann 2005; Touma & Palme 2005). Higher glucocorticoid metabolite levels during breeding have been reported in several studies in mammalian species (e.g. white-tailed deer: Moen et al. 1982; spotted hyenas: Goymann et al. 2001; European pine marten: Barja et al. 2007; felid species: Brown & Wildt 1997). Due to the polygynous mating system of the common vole (Boyce & Boyce 1988), the strong competition among breeding males in order to breed as many females as possible could generate a rise in intrasexual aggressive behaviour with an increase in glucocorticoid levels. Otherwise, glucocorticoids play an important role in

metabolism (Tataranni et al. 1996) and it is known that, in mammals, breeding females undergo a series of changes in metabolism during pregnancy and lactation due to the request for maternal investment (Bauman 2000; Strier et al. 2003; Reeder & Kramer 2005). These metabolic changes could explain the encountered FCM variation between breeding and non-breeding individuals.

Part of the variation found in FCM concentrations was explained by the abundance of common voles. Individuals captured within crops or seminatural field margins with higher abundance showed higher levels of FCM. These results match up with other studies where high population densities have been reported to be a main factor causing increased glucocorticoid metabolite levels in several species (Goymann & Wingfield 2004; Raouf et al. 2006). These higher levels of FCM could be explained because high densities are likely to generate an increase in intraspecific competition with the consequent decrease in the availability of food, refuges or partners, what strongly affects small rodent populations (Lemen & Clausen 1984; Byrom et al. 2000).

Our results show some evidence that highly managed habitats such as crops in agricultural landscapes are associated with higher glucocorticoid levels in common voles. These results seem to match with those found in other studies where individuals subjected to a higher human pressure (e.g. tourism, winter sports, snowmobile activity…) showed higher levels of faecal glucocorticoids (Millspaugh et al. 2001; Creel et al. 2002; Barja et al. 2007; Thiel et al. 2011; Piñeiro et al. 2012; Zwijacz-Kozica et al. 2013). As physiological stress can also affect animal fitness (Möstl and Palme, 2002), understanding how human manipulations of habitats are associated with elevated glucocorticoid levels in free-living animals could help to better understand other changes with a slower response in time (i.e. behavioural changes, changes in species composition and/or abundance) observed in animal populations. Although we carried out a snapshot study by a single sampling and despite the lack of comparison of faecal and plasma glucocorticoid levels and the excretion time of glucocorticoids in this species, this is the first study done in this direction. So, our results might therefore encourage further research broadening the spatial and temporal framework of the stress-habitat-human relationship for wild species.

PAPER VI

ARE MOTROWAYS POTENTIAL STRESSORS OF ROADSIDE WOOD MICE (*Apodemus sylvaticus*)?

ABSTRACT

Linear infrastructures represent one of the most important human impacts on natural habitats and exert several effects on mammal populations. Motorways are recognized as a major cause of habitat fragmentation and degradation and of biodiversity loss. However, it is unknown whether motorways lead to increased physiological stress reactions in wild animal populations. We analysed faecal corticosterone metabolites (FCM) in wild populations of wood mice (*Apodemus sylvaticus*) living in a well-preserved Mediterranean agro-pastoral woodland at different distances (verge, 500 m and 1000 m) from the AP-51 motorway in Spain. Wood mice were captured with Sherman live traps, and fresh faecal samples from 424 individuals were collected and analyzed in the laboratory. The quantification of FCM was performed by a 5α-pregnane-3β,11β, 21-triol-20-one enzyme immunoassay. Results showed that females had higher FCM levels than males, and these levels were higher in breeding females. In addition, FCM levels were positively correlated with body weight of individuals. Wood mice captured where cattle were present showed higher FCM levels than individuals living where cattle were not detected. FCM levels were higher in non-breeding individuals living close to the motorway compared with FCM levels in those individuals captured further from the motorway. This is the first study showing evidence of the motorways' impact on physiological stress reactions in wild wood mice populations. Understanding how free-living animals are influenced by human interventions could help to understand other subtle changes observed in wild animal populations. Since mice are used world-wide as research models these results could open new perspectives testing human influence on the natural environment and trade-offs of species in degraded ecosystems.

Keywords: faecal corticosterone metabolites, glucocorticoids, human impacts, motorways, physiological stress reactions, road ecology

INTRODUCTION

Landscapes are often dissected by an extensive network of linear elements like roads and motorways which occupy huge areas resulting in land use conversion and becoming one of the most extended human impacts in natural habitats (Spellerberg 2002; Forman et al. 2003). They can affect wild animal populations directly due to road mortality (Lodé 2000; Clevenger et al. 2003) and indirectly, due to the reduction in the connectivity of the landscape because of the habitat fragmentation and to the loss and degradation of habitats (Saunders et al. 2002; Coffin 2007). Alterations to light, moisture and wind regimes due to the creation of edges, and pollution from traffic, e.g., light, noise, and chemical, are all perturbations linked to roads accompanying the infrastructure along its whole live (van der Ree et al. 2011). Thus, ecological effects arising from them can determine the viability of local wildlife populations even if their short-term impact is small (Lodé 2000; Saunders et al. 2002).

Several studies have been performed to determine and understand the effects of roads and motorways on wild animal populations. Most of these studies have focused on medium size to large mammals (McLellan & Shackleton 1988; Thurber et al. 1994; Jones 2000; Lodé 2000; Roedenbeck & Voser 2008) and only few studies have been carried out with small mammals (Goosem 2001; Rico et al. 2007; McGregor et al. 2008). However, although motorways have negative effects on wild mammalian populations causing a physical and/or behavioral barrier effect (Klein 1971), no studies have documented their effects on other aspects, e.g. the effect of motorways on physiological stress reactions in wild mammal populations is unknown.

For the assessment of physiological effects, glucocorticoids (GC) have been used as indicators of stress (Harper & Austad 2001; Möstl & Palme 2002; Good et al. 2003; Ylönen et al. 2006; Götz & Stefanski 2007; Sheriff et al. 2011). Especially in wildlife studies, faecal cortisol/corticosterone metabolites (FCM) have been utilized as a suitable, non-invasive measure of GC levels to evaluate responses during stressful stimuli and the endocrine status in an increasing number of mammalian species (Touma & Palme 2005; Barja et al. 2007; Monclús et al. 2009; Sheriff et al. 2011).While short-term GC secretion is related to animal adaptive responses to a stressor (Wingfield & Romero 2001), chronically elevated and prolonged high GC levels caused by environmental or human perturbations can lead to serious consequences on

reproduction, the immune function, growth, survival and fitness (Munck et al. 1984; Sapolsky et al. 2000; Möstl & Palme 2002; Stewart 2003; Romero 2004) .Different studies performed in natural conditions have correlated GC levels to human perturbations (Millspaugh et al. 2001; Creel et al. 2002; Arlettaz et al. 2007; Barja et al. 2007; Thiel et al. 2008; Thiel et al. 2011; Piñeiro et al. 2012). So, since motorways are human made interventions and involve the modification of natural ecosystems, they could be perceived by animals as stressors evoking a stress response in wild populations.

OBJECTIVES

Therefore, the aim of the present study was to test the hypothesis that human disturbance by motorways is a potential stressor that may affect GC concentrations causing physiological stress reactions in small mammals like the wood mouse (*Apodemus sylvaticus*). Most ecological effects are continuous along a road but their extension or impact over wild populations depend on the closeness to the road, and this significant impact varies from a few meters to a few kilometers (Reck & Kaule 1993; Forman 1995). Therefore, if wood mice perceive vehicles as a real disturbance, then we predict that the FCM levels would vary in relation to the distance to the motorway. We would expect higher FCM levels in those individuals living closer to the motorway subjected to a major degree of environmental change and human disturbance due to traffic. However, if traffic is not a direct disturbance and roads are favorable habitats, FCM levels should be similar regardless the distance to the motorway.

MATERIAL AND METHODS

Study Area

The study took place along a stretch of the AP-51 motorway in Ávila (central Spain) and it was carried out under the permit issued by the Dirección General del Medio Ambiente, Junta de Castilla y León (Reference: CML/mjg EP/CYL/424/2009) with permission from the land proprietors of El Tabladillo estate and landowners from Mediana de Voltoya and Ojos Albos. The road is fenced and it has two lanes in each direction, with a mean traffic volume of 8396 vehicles per day. The study area is included in the European Natura 2000 Network as a Site of Community Importance (SCI ES-4110103 Encinares de los ríos, Adaja and Voltoya). The surrounding

environment is dominated by holm oak (*Quercus ilex*) woodlands, mixed with open holm oak grazing woodlands devoted to low intensity livestock ranching, fallow areas and scrub and unirrigated cereal crops. The study area has a typical Mediterranean climate with an annual mean temperature of 10º C, a mean annual precipitation of 364mm and two-month long dry season in July–August. The location ranges from 1050 to 1250 m above sea level.

Animal Trapping and Data Collection in the Field

Live trapping was conducted in February and April of 2010 along five kilometres of the AP-51 (UTM 30 T 373259 4510571– 30 T 368053 4507625) where four 1250 m sectors were selected. In each sector, three bands were established at increasing distances (0-50 m, 500 m and 1000 m) in line with the road (Ruiz-Capillas et al. 2013). The three distance bands were sampled simultaneously to avoid the effects of weather conditions and moonlight on small mammals' activity (Clarke 1983; Vickery & Rivest 1992). Thirty Sherman live traps (23x8x9 cm) were set up in each band with 10-15 m of separation between them, for three consecutive nights and with a total effort of 2160 traps-night. Traps were reviewed twice-daily every 8-10 hours, at dawn and dusk, to minimize the time that animals were kept inside. Bread fried in rancid oil was used as bait and cotton nesting material was provided inside each trap as bedding to protect captives from low temperatures. In addition, traps were hidden under vegetation cover to protect animals from adverse weather conditions and to avoid detection by predators. Captured animals were marked with subcutaneous sterile Trovan ID100 passive transponders to identify recaptures in order to achieve sample independence. Individuals were weighted with a hand-held scale. Sex and breeding condition of individuals were determined following the method of Gurnell & Flowerdew (1994). The distance between the clitoris and anus in females is smaller than the distance between the penis and anus in males. Breeding adult males present the testicles enlarged quite markedly and usually descend into the scrotal sac while in breeding adult females the nipples on the abdomen and thorax are noticeable and the vaginal membrane appears perforated. To reduce disturbance to animals, all captured individuals were handled as fast as possible and they were released in the same place of capture. Traps were cleaned with clean water to avoid the mixing of different samples and then reactivated.

Since vegetation characteristics and cattle activity may affect small mammals' behaviour and/or abundance (Orrock et al. 2004; Torre et al. 2007; Muñoz et al. 2009), these variables were recorded as well as the distance-band. Vegetation cover and height were measured around each trap (area of 5 m radius) by two experienced observers. The plant cover (in %) was visually estimated, and the mean height of the herbaceous and shrub stratum (in cm) was measured by placing a stick vertically on the ground. Cattle activity (presence or grazed field) was coded as one when there were signs of cattle activity and otherwise it was recorded as zero.

Faecal Samples Collection

Faeces were collected from traps where a single wood mouse was captured. We only collected fresh faecal samples (i.e. with a soft texture and not dried) in order to prevent degradation of steroids by microorganisms (Möstl et al. 2005). To avoid cross contamination faecal samples from traps where urine was detected were excluded. FCM appear in faeces about 10 h after an ACTH injection (median: 10 h, range: 8–12h; Touma et al. 2004), so traps were reviewed within 10 hours to avoid any possible effect of the capture itself on FCM levels. Circadian rhythm is supposed to have an important influence in excretion patterns (Touma et al. 2003; Touma et al. 2004), so we considered only the fresh samples collected during the early morning checking. Faecal samples were stored in the freezer at -20º C until analysis.

Measurement of Faecal Corticosterone Metabolites (FCM)

Extraction of FCM from the faeces was performed as described previously Touma et al. (2003) and Palme et al. (2013). Briefly, each faecal sample was homogenized and 0.05 g were weighed and mixed with 1 ml of 80% methanol in an eppendorf tube. Samples were shaken for 30 min on a multivortex and then centrifuged (15 min at 2500 *g*). Supernatants obtained were diluted 1:10 with assay buffer and stored at -20ºC until analysis. FCM were analyzed using an already established 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) measuring metabolites with a 5α-3β,11β diol structure. This method was previously developed and validated for mice by (Touma et al. 2003; Touma et al. 2004).

Statistical Analyses

We used a General Linear Model (GLM) to analyze factors influencing FCM levels. The response variable, i.e. concentrations of FCM, was $\log_{10}$ transformed to reach normal distribution and homocedasticity. Categorical predictors were band´s distance (0-50 m / 500 m / 1000 m), sex (male / female), breeding condition (breeding / non-breeding), and cattle (presence or habitat grazed / no signs), while weight of individuals was fitted as covariable. Plant cover and height were not included in the statistical model since their values were mainly related to cattle grazing as denoted by exploratory analyses. Thus, ungrazed sites showed a denser and taller grass and shrub layer than grazed ones. Differences in FCM levels between groups were analyzed for significant interactions in the GLM through Tukey's honestly significant difference (HSD) post-hoc tests carried out on their marginal means. Results were considered significant at $p < 0.05$. Data are given as mean ± standard error (SE). We used the SPSS 15.0 statistical software (SPSS Inc, Chicago, IL, U.S.A.) to perform the GLM, and Tukey HSD tests were computed in a worksheet according to formulae (Quinn & Keough 2002).

RESULTS

FCM levels were analyzed in fresh faecal samples from a total of 424 different wood mice (Table 14). The statistical model (Table 15) showed that significant factors explaining the variation found in FCM concentrations were sex, breeding condition, body weight of individuals and cattle. In addition, the interactions between sex*breeding condition and band*breeding condition resulted in a significant influence on FCM levels. Other interactions were not statistically significant.

Table 14. Number of captures in the different distance bands in relation to sex and breeding condition of individuals.

	Distance bands					
	0-50 m		500 m		1000 m	
Number of captures	187		94		143	
Males / Females	88 / 99		42 / 52		82 / 61	
Breeding / Non-breeding	10/78	29/70	4/38	11/41	2/80	12/49

Table 15. Results of the General Linear Model testing the effects of individual and environmental variables on faecal corticosterone metabolites in wood mouse.

Factor	F	df	*P*
Band	0.326	2	0.722
Sex	11.730	1	0.001
Breeding condition	11.711	1	0.001
Weight of individuals	14.945	1	0.000
Cattle	3.944	1	0.048
Sex * Breeding condition	4.786	1	0.029
Band*Breeding condition	5.166	2	0.006
Band*Sex	2.241	2	0.108
Band*Cattle	1.608	1	0.205
Cattle*Sex	2.004	1	0.158
Cattle*Breeding condition	2.174	1	0.141
Band*Cattle*Sex	0.323	1	0.570
Band*Cattle*Breeding condition	0.035	1	0.852
Band*Sex*Breeding condition	0.634	2	0.531
Cattle*Sex*Breeding condition	3.355	1	0.068

FCM levels differed between sexes, concentrations of FCM were higher in females (4467 ± 485 ng/g dry faeces) than in males (2057 ± 235 ng/g dry faeces) ($F_{1,424}$ = 11.73, P = 0.001). Breeding individuals showed higher FCM levels (6609 ± 978 ng/g dry faeces) compared to the non-breeding ones (2601 ± 255 ng/g dry faeces) ($F_{1,424}$ = 11.71, P = 0.001). Nevertheless, the interaction between sex*breeding condition ($F_{1,424}$ = 4.79, P = 0.029) showed that differences due to breeding condition were only significant for females (Tukey´s HSD, $P < 0.01$), but not for males (Tukey´s HSD, $P > 0.05$) (Fig. 19). In addition, FCM correlated positively with body weight of animals ($F_{1,424}$ = 14.95, $P < 0.0001$). Cattle had a significant effect on FCM levels ($F_{1,424}$ = 3.94, P = 0.048), individuals captured where cattle (presence or habitat grazed) were detected shown higher FCM levels (3779 ± 451 ng/g dry faeces) than individuals captured where no signs of cattle were recorded (3042 ± 344 ng/g dry faeces).

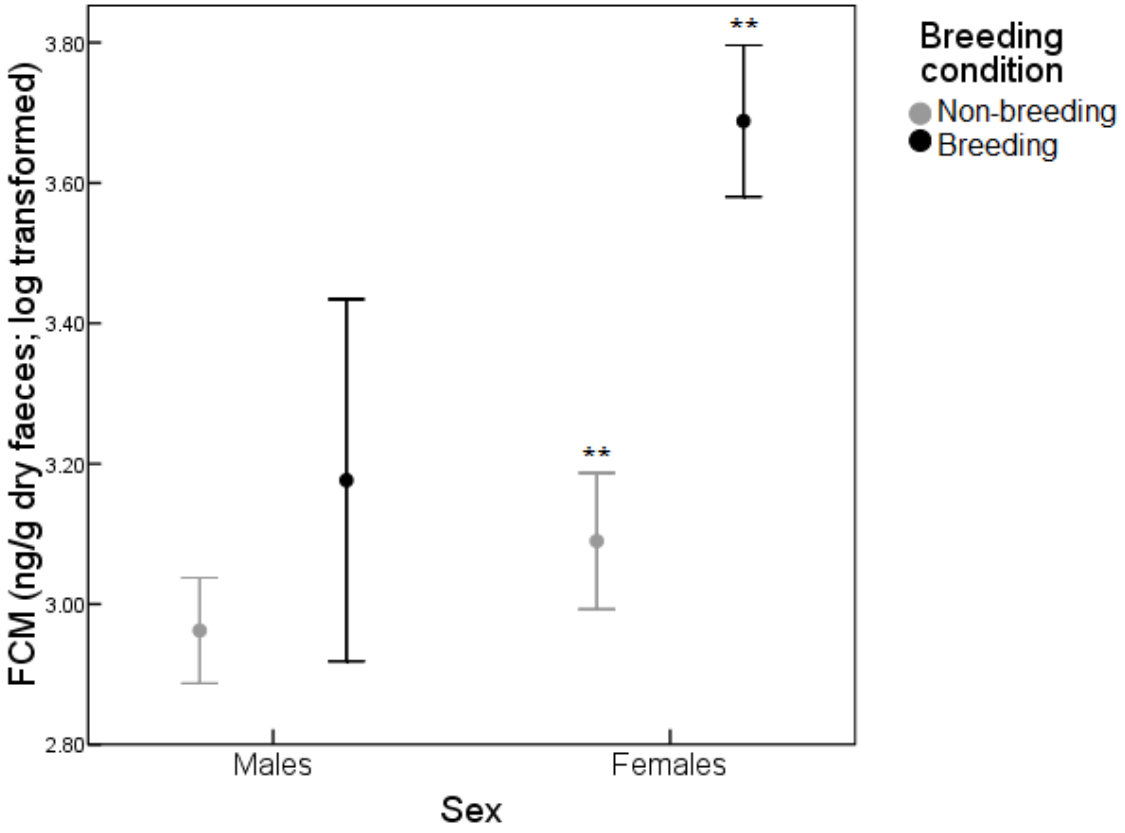


Figure 19. Comparison of FCM concentrations (ng/g dry faeces; mean ± SE) according to breeding condition for males and females. Asterisks indicate significant differences (** $p < 0.01$).

The significant interaction between band*breeding condition showed that FCM levels varied according to the distance to the motorway, however, significant differences between distance-bands depended on breeding condition (Fig. 20). FCM levels were similar in the three distance-bands for breeding individuals (Tukey´s HSD, $P > 0.05$) but they showed significantly higher values in the non breeding individuals from the 0-50m band compared with those captured in the 1000m band (Tukey´s HSD, $P < 0.05$). FCM values for non-breeding individuals captured at 500m were midway between those of individuals captured beside the road and at the 1000m band.

Figure 20. Log-transformed concentrations (mean ± SE) of faecal corticosterone metabolites (FCM, ng/g dry faeces) in breeding and non-breeding individuals in relation to the distance to the motorway. Significant differences are indicated by asterisks (* $p < 0.05$).

DISCUSSION

Glucocorticoids relationship with sex and breeding condition has yielded ambiguous results (Touma et al. 2003; Harper & Austad 2004; Touma et al. 2004; Schradin 2008; Busch & Hayward 2009). We found differences in FCM levels between both sexes, females showing higher levels of FCM than males. The same situation was reported for other rodent species (Touma et al. 2004; Navarro-Castilla et al. 2014a) and this difference could be partly due to differences in the metabolism of glucocorticoids between both sexes (Touma et al. 2003). Higher glucocorticoid levels during breeding season have been reported in several studies performed in different mammalian species (Moen et al. 1982; Goymann et al. 2001; Romero 2002; Barja et al. 2007; Dantzer et al. 2010). However, in our case we only found this pattern for females but not for males, probably because the low sample size of males. Nevertheless, a possible explanation could be that testosterone in breeding males can suppress glucocorticoids, whereas high

oestrogen levels during lactation and pregnancy should increase them (Handa et al. 1994). Glucocorticoids play an important role in metabolism (Tataranni et al. 1996) and it is known that breeding females undergo a series of metabolic changes during pregnancy and lactation due to their maternal investment (Bauman 2000; Strier et al. 2003; Reeder & Kramer 2005). In addition, reproduction costs reduce longevity in mice and other species (Russell 1966; Roff 1992), especially in females (Penn & Smith 2007). So, reproduction could be probably not particularly stressful for males since their investment is usually limited to fertilization, whereas socio-sexual aggression may be a normal component of female´s cost of reproduction and females, through gestation and lactation, invest heavily in the offspring's survival. Therefore, the difference found in FCM levels between males and females and during breeding could be explained by the mix of different metabolism patterns and their different constraints on reproduction.

We found that FCM levels were positively related with body weight of individuals. Since in this species weight of individuals is closely related with animal´s age (Gurnell & Flowerdew 1994), our results suggest adult individuals showing higher levels of physiological stress reactions than young individuals. Elevated glucocorticoid secretion promotes physiological and behavioral responses that enhance an adult animal's ability to cope with stressors and other challenges to homeostasis (Wingfield 1994), whereas less well understood is whether similar glucocorticoid-mediated responses occur during other different stages of life (Schwabl 1999). These results could be explained by age-related alterations (Hauger et al. 1994). Alternatively this difference found in FCM levels between young and adult animals could also be due to differences in metabolism and excretion of glucocorticoids rather than differences in plasma corticosterone levels (Touma & Palme 2005).

The effect of cattle on FCM levels could be explained because grazing by herbivores reduces vegetative cover drastically (Elton 1939; Birney et al. 1976) and this has an important impact on small mammal communities (Tester & Marshall 1961; Putman et al. 1989; Heske & Campbell 1991; Eccard et al. 2000). Small mammals perceive dense vegetation as good protection (Tchabovsky et al. 2001) since the reduction of vegetation cover exposes small mammals to increased predation risk (Preston 1990; Sheffield et al. 2001; Orrock et al. 2004). Thus, our results suggest that habitats without cattle, therefore with more grass and shrub cover, could be considered

as better quality habitats with a higher protection from predators and therefore lower FCM values.

The higher FCM values found in breeding individuals in the three distance-bands showed that breeding condition has a huge influence on FCM as we have discussed above. These results suggest that the effect of motorway on FCM could be less important when there are other stronger factors like breeding status affecting to the individuals. However, the higher FCM levels found in non-breeding individuals living close to the motorway could be explained by the alteration of the natural habitat and the perturbation of the motorway. An effect that could be diluted as distance to the motorway increases, with levels still higher at 500 m than at 1000 m. Road maintenance and traffic aggravate edge effects on the surrounding environment by noise and pollution. Vehicle´s emissions cause atmospheric pollution and consequent ecological effects for wildlife (Winner 1994) and traffic noise is known to be an important source of disturbance (Frid & Dill 2002; Parris et al. 2009). However, visual disturbance, the barrier effect and pollutants extended outward only a short distance compared with traffic noise that spreads far into the environment. Several studies have shown that effect-distances are sensitive to traffic density, traffic speed or the type of habitat being higher the effects in the proximity of roads and varying from 300 m to 900 m (Reijnen 1995; Reijnen et al. 1995). In our case, both traffic (density and speed) and habitat type was similar along the five kilometres selected, so this distance effect of traffic noise could explain the variation found in FCM levels at different distances from the motorway. Many possible reasons exist for the effects of traffic noise on wildlife, likely hypotheses include hearing loss, altered behavior, interference with communication during breeding activities, differential sensitivity to different frequencies, and deleterious effects on food supply or other habitat attributes (Andrews 1990; Reijnen 1995; Reijnen et al. 1995; Wasser et al. 1997). Taken together, our results suggest that the negative effects of motorways, through the barrier effect or the traffic noise, seem to be an important factor of perturbation for wildlife communities evoking an increase in FCM levels.

According to our results, there is a first evidence of motorways increasing stress reactions in a small mammal species. Wood mouse is known by its great ability to use a wide range of open habitats including urban habitats, forest, meadows, forest,

grasslands and agricultural crops (Kikkawa 1964; Pollard & Relton 1970; Green 1979; Angelstam et al. 1987; Gorman et al. 1993) and is, therefore, less likely to be dramatically affected by environmental changes. However, several studies have shown that small mammals are hardly affected by the barrier effect of roads (Rico et al. 2007; McGregor et al. 2008) and our results demonstrate that in this species motorways are acting like a stressor factor, and even this could be for other animal species too. Our study has implications for the effectiveness of different possible mitigation strategies to reduce the impact of roads on mammal species being the major conservation objective focused in the conservation of large roadless areas (Crist et al. 2005). To our knowledge, this is the first attempt to test the physiological stress reaction in wood mice caused by the perturbation of motorways. Our results are of great concern due to the high proliferation of roads in the landscape over recent decades. In combination with other studies demonstrating complex and negative effects of roads on other species, affecting populations' viability, our results might be relevant since FCM levels may indicate that an animal is stressed even if obvious behavioral changes cannot be detected (Walker et al. 2005). These results shown that FCM measurement can provide a powerful tool for addressing and evaluating the impacts of human disturbance on wildlife populations. However, the biological responses evoked to cope with a stressor vary among species, age, experience and physiological status (Moberg 1985; Moberg 2000). So, further research should be performed in order to study this human-based stressor in other wild animal species.

CONCLUDING REMARKS

Risk of predation

1. Wood mice recognized and avoided their main predators, the red fox and the common genet, in the study areas. However, they did not respond to the faecal odour of an unknown potential predator, the European pine marten, maybe due to the lack of a common historical evolution between both species suggesting that prior experience with predators is a key factor.

2. Wood mice also responded to indirect predation risk by decreasing the number of captures during the increased illumination periods (full moon) because these conditions make them more visible and, therefore, more vulnerable to predators.

3. Predation risk influenced foraging activity which was reduced in presence of predator faeces (common genet). Furthermore, food intake decreased during increased illumination nights (full moon) and it was lower in traps treated with predator faeces (red fox and common genet).

4. The behavioural responses mentioned above were influenced by individual characteristics. Thus, common genet avoidance was significantly higher by non breeding adult males. Equally, breeding individuals reduced food intake when they faced red fox and common genet faeces. This suggests that wood mice responses to predation risk vary depending on individual characteristics probably due to the previous experience and the balance of cost-benefits in each particular situation, at a given place and time, and by each individual.

5. Females, breeding individuals and adults showed higher faecal corticosterone metabolite (FCM) levels. However, no physiological stress responses were found due to direct (predator faeces) or indirect (moon light) predation risk. This suggests that these cues could not be enough reliable to experience physiological stress responses since prey species daily face the risk of predation and, therefore, such continuous responses could be energetically costly and pathological for individuals.

Interspecific competition

6. Wild ungulates increased soil compaction and decreased vegetation height and cover outside the exclosure areas.

7. Algerian mice abundance was higher inside the exclosures where vegetation features (cover and height) were greater and soil compaction was lower. Habitat selection was for smaller trees with greater vegetation cover, which increases protection against predators.

8. Algerian mice females showed higher FCM concentrations than males because of sex differences in the metabolism of glucocorticoids. Increased physiological stress responses inside exclosure areas were related to the higher mice abundance whereas in the grazed areas the increase in FCM levels was associated to the higher soil compaction caused by ungulates.

Human disturbances

9. Common voles were less captured inside crops due to the avoidance of agricultural practices. Females and breeding individuals showed higher FCM levels. In addition, abundance of voles was positively correlated with FCM levels. Individuals living inside crops exhibited higher physiological stress levels due to the human disturbance. Field margins supported more individuals with lower FCM levels associated to the lesser disturbance and higher protection against predators.

10. Wood mice inhabiting closer to the motorway showed increased physiological stress responses due to the associated disturbances (e.g. noise and traffic).

CONCLUSIONES

Riesgo de depredación

1. Los ratones de campo reconocieron y evitaron a sus depredadores, el zorro y la gineta, en las dos áreas de estudio. Sin embargo, no respondieron al olor fecal de un depredador desconocido, la marta europea, quizás por la inexistencia de una historia evolutiva común entre ambas especies lo que sugiere que la experiencia previa con los depredadores es un factor clave.

2. Los ratones de campo respondieron al riesgo de depredación indirecto, disminuyendo el número de capturas durante los días de mayor iluminación (luna llena) ya que estas condiciones les hacen más visibles y, por tanto, más vulnerables a los depredadores.

3. El riesgo de depredación influyó en el tiempo dedicado a la búsqueda de alimento siendo éste menor en presencia de heces de depredador (gineta). Del mismo modo, la ingesta de alimento se redujo en los periodos de mayor iluminación (luna llena) y en las trampas tratadas con heces de depredador (zorro y gineta).

4. Las respuestas comportamentales mencionadas previamente estuvieron influenciadas por características individuales. Así, los machos subadultos no reproductores evitaron más las heces de gineta que el resto de individuos. Igualmente, los individuos reproductores redujeron la ingesta de alimento en presencia de heces de zorro y de gineta. Estos resultados sugieren que las respuestas del ratón de campo al riesgo de depredación directo varían en relación a características individuales, probablemente debido a la experiencia previa y a la valoración de los costes-beneficios en cada situación particular y en un lugar y momento determinados.

5. Las hembras, los individuos reproductores y los adultos mostraron mayores niveles de metabolitos de corticosterona fecal (MCF). Sin embargo, no se encontraron respuestas de estrés fisiológico debido al riesgo de depredación directo (heces de depredador) e indirecto (iluminación lunar). Esto parece sugerir que estas señales no son

suficientes para activar las respuestas de estrés fisiológico, pues las presas se encuentran diariamente sometidas a riesgo de depredación y, por tanto, activar dichas respuestas continuamente podría ser muy costoso energéticamente y patológico para los individuos.

Competencia interespecífica

6. Los ungulados silvestres aumentaron la compactación del suelo y redujeron la altura y cobertura de la vegetación fuera de las áreas de exclusión de ungulados.

7. La abundancia de ratón moruno fue mayor dentro de las exclusiones donde encontramos mayor cobertura y altura de la vegetación y menor compactación del suelo. Además, el ratón moruno seleccionó árboles más pequeños y con mayor cobertura de vegetación, la cual ofrece más protección frente a los depredadores.

8. Las hembras de ratón moruno presentaron mayores niveles de MCF que los machos debido a diferencias sexuales en el metabolismo de los glucocorticoides. El incremento de las respuestas de estrés fisiológico dentro de las exclusiones de ungulados se relacionó con el aumento de la abundancia de ratón moruno. Sin embargo, fuera de las exclusiones el incremento de los niveles de MCF se asoció con la mayor compactación del suelo.

Perturbaciones humanas

9. El topillo común fue menos capturado dentro de los cultivos, evitando las perturbaciones provocadas por las prácticas agrícolas. Las hembras y los individuos reproductores mostraron mayores niveles de MCF. Además, la abundancia de topillos estuvo relacionada positivamente con el aumento de las concentraciones de MCF. Los individuos capturados dentro de los cultivos presentaron mayores concentraciones de MCF. No obstante, en los márgenes se capturaron más individuos y éstos mostraron menores niveles de estrés debido a una menor perturbación del hábitat y mayor protección frente a depredadores.

10. Los ratones de campo capturados en las proximidades de la autopista mostraron mayores niveles de MCF debido a las perturbaciones asociadas a ésta (p.e. ruido, tráfico, entre otras).

IMPLICATIONS FOR THE CONSERVATION

Based on all these results, we can conclude that small mammals seem to respond by altering their behaviour when facing daily environmental or human disturbances. However, they only experienced physiological stress responses due to factors like the interspecific competition with ungulates (directly by soil compaction and indirectly by increasing mice abundances) and human disturbances.

The results from the predation risk experiments showed that wood mice avoided predator faeces and increased illumination. Therefore, both factors combined could be used as management tools for controlling small mammals activity in agricultural crops. These measures would probably prevent undesirable pests, being also a lesser harmful alternative than poisoning and its proven negative consequences for this and other wild animal species.

Our results revealed interspecific competition with ungulates and human disturbances reduced small mammal populations and increased physiological stress levels. Our studies also suggest that natural or less altered patches may attenuate these effects. These findings are of great value to develop management strategies for the conservation of small mammal populations.

Small mammals are good indicators of environmental impacts and they play an important role either as seed dispersers or as a major resource for many predators, hence, knowing how they cope with environmental and human factors could be crucial for developing effective management and conservation strategies in these but also in other free-ranging species.

IMPLICACIONES PARA LA CONSERVACIÓN

En base a estos resultados, podemos concluir que frente a las perturbaciones ambientales y humanas los micromamíferos responden alterando su comportamiento. Sin embargo, la respuesta de estrés fisiológico únicamente se activó debido a factores como la competencia interespecífica con ungulados (directamente por la compactación del suelo e indirectamente por el aumento en las abundancias de ratón) y las perturbaciones humanas.

Los resultados de los experimentos de riesgo de depredación mostraron que los ratones de campo evitaron las heces de los depredadores y el incremento de iluminación. Por tanto, la combinación de ambos factores podría utilizarse como una herramienta de manejo para controlar la actividad de los micromamíferos en los cultivos agrícolas. Estas medidas podrían prevenir las plagas siendo una alternativa menos dañina al uso de venenos y sus ya demostradas consecuencias negativas para ésta y otras especies silvestres.

Nuestros resultados revelaron que la competencia interespecifica con ungulados silvestres y las perturbaciones humanas reducían la abundancia de micromamíferos e incrementaban los niveles de estrés fisiológico. Dichos estudios también sugieren que las zonas menos alteradas pueden atenuar estos efectos. Estos hallazgos, sin duda, resultan de gran importancia para los gestores del medio natural, ayudando a elaborar estrategias para la conservación de las poblaciones de micromamíferos.

Por último, cabe destacar que los micromamíferos son buenos indicadores de los impactos en el medio natural y juegan un papel importante como dispersores de semillas y como fuente de alimento para muchos depredadores. Por tanto, conocer cómo pueden verse afectados por diferentes factores, tanto ambientales como humanos, puede ser crucial para desarrollar planes de gestión y estrategias de conservación efectivas en éstas y otras especies.

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APPENDIX I

LIST OF PAPERS

Chapters of this doctoral thesis are published or under review in the following journals included in the *Science Citation Index*:

Navarro-Castilla, Á. & Barja, I. (2014). Antipredatory response and food intake in wood mice (*Apodemus sylvaticus*) under simulated predation risk by resident and novel carnivorous predators. *Ethology* 120: 90-98.

Navarro-Castilla, Á. & Barja, I. (2014). Does predation risk, through moon phase and predator cues, modulate food intake, antipredatory and physiological responses in wood mice (*Apodemus sylvaticus*)? *Behavioral Ecology and Sociobiology* 68: 1505-1512.

Navarro-Castilla, Á., Barja, I. & Díaz, M. Behavioral and physiological stress responses to simulated predation risk in wild wood mice. *Journal of Mammalogy* (under review).

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Navarro-Castilla, Á., Barja, I., Olea, P. P., Piñeiro, A., Mateo-Tomás, P., Silván, G. & Illera, J. C. (2014). Are degraded habitats from agricultural crops associated with elevated faecal glucocorticoids in a wild population of common vole (*Microtus arvalis*)? *Mammalian Biology* 79: 36-43.

Navarro-Castilla, Á., Mata, C., Ruiz-Capillas, P., Palme, R., Malo, J. E. & Barja, I. (2014). Are motorways potential stressors of roadside wood mice (*Apodemus sylvaticus*) populations? *PLoS One* 9:e91942.